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N.B.: When citing this work, cite the original article.

The original publication is available at www.springerlink.com:

Andreas Lewander, Jingfang Gao, John Carstensen, Gunnar Arbman, Hong Zhang and Xiao-Feng Sun, NF-kappa B p65 phosphorylated at serine-536 is an independent prognostic factor in Swedish colorectal cancer patients, 2012, International Journal of Colorectal Disease, (27), 4, 447-452.

<http://dx.doi.org/10.1007/s00384-011-1356-8>

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Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-76610>

NF- κ B p65 Phosphorylated at Serine-536 Is an Independent Prognostic Factor in Swedish Colorectal Cancer Patients¹

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Running Title: NF- κ B p65 in Colorectal Cancer Patients

¹The study was supported by grants from the Swedish Cancer Foundation, Swedish Research Council and the Health Research Council in the South-East of Sweden.

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Abstract

NF- κ B transcription factor protein family has diverse cellular and biological functions, and post-translational modification is important to regulate these functions. An important site of phosphorylation of NF- κ B p65 subunit is at Serine-536 (phospho-Ser536-p65), and this phosphorylation is involved in regulation of transcriptional activity, nuclear localization and protein stability. In this study, we investigated expression of phospho-Ser536-p65 in colorectal cancers and its relationships with clinicopathological factors. The expression of phospho-Ser536-p65 was examined by immunohistochemistry in 203 primary colorectal cancers, 156 normal mucosa specimens and 18 metastases in the lymph nodes. The expression of phospho-Ser536-p65 increased from normal mucosa to primary tumor ($p < 0.0001$). Further, the increased expression of phospho-Ser536-p65 in the cytoplasm of the primary tumors correlated with worse survival of the patients independently of gender, age, tumor location, stage and differentiation ($p = 0.04$, hazard ratio 1.89, 95% CI 1.03-3.47). The NF- κ B p65 subunit phosphorylated at Serine-536 is an independent prognostic factor in colorectal cancer patients.

Key words: NF- κ B, Survival, Colorectal cancer, Immunohistochemistry.

Introduction

Colorectal cancer is one of the most common forms of cancers after prostate and breast cancer in the Western world. Despite extraordinary progress in survival due to improved diagnosis and especially treatment, many patients still suffer from recurrence and eventually die from the cancer. There is significant variation in the response to the treatments and survival among patients even though they have tumour in the same stage [1]. Therefore, it is very important to search for more biological markers to identify patient survival and the response to treatments.

NF- κ B transcription factor protein family is involved in numerous cellular activities that, if not kept in balance, will transform the cell into a cancer cell [2,3]. Five members of the family have been found in human cells, RelA (p65), p105/p50, p100/p52, RelB and c-Rel. The most common form in human cells is p65/p50 heterodimer [2,4,5]. The regulation of the NF- κ B protein family is very important. Dimers of NF- κ B are kept in the cytoplasm in the cells in an inactive state by binding to inhibitory proteins. Upon activation, inhibitory proteins are degraded and the proteins are translocated into the nucleus where they exert their effect. The regulation also occurs at the posttranslational level, where protein phosphorylation of the different subunits is a very important mechanism of regulation. Several different phosphorylation sites on the subunits have been discovered. An important site of phosphorylation of p65 subunit is at Serine-536 (phospho-Ser536-p65), and this phosphorylation is involved in regulation of transcriptional activity, nuclear localization and protein stability [6-9]. Studies have shown that the phospho-Ser536-p65 can be found in both the cytoplasm and the nucleus depending on

the stimulus, and the phosphorylation is probably also cell-type specific [4,10-12].

In the light of recent knowledge of the importance of regulation of NF- κ B activity through phosphorylation we wanted to investigate whether expression of phospho-Ser536-p65 was related to colorectal cancer development, especially to clinicopathological variables, including survival, in patients.

Materials and methods

Patients

Formalin-fixed paraffin-embedded tissue blocks were obtained from 203 patients with primary colorectal adenocarcinoma as the patients underwent surgical resection at Linköping Hospital or Vrinnevi hospital between 1972 and 2004. The study also included 156 normal mucosa specimens (of them 128 were matched with the primary tumors) taken from either the margin of distant resection or adjacent to the primary tumors, and 18 metastases in the lymph nodes (12 of them were matched with the primary tumors). The patients' gender, age, tumor location, stage and differentiation (better differentiation including well and moderate differentiation and poorer differentiation including poor differentiation, mucinous and signet-ring cell carcinomas) were obtained from surgical and/or pathological records at Linköping and Vrinnevi Hospitals. All patients were followed-up, and the mean period of the follow-up was 77 months (median, 56 months, and range, 0.06-288 months), the data were received/confirmed from/by surgeons and oncologist as well as the staff at Swedish Cancer Register Center, and the follow-up information was updated at least annually. The survival analyses were based on overall survival.

Immunohistochemistry

Five-micrometer sections were deparaffinised in xylene and rehydrated in graded ethanol. The method for antigen retrieval we used was high-pressure cooking in 0.01 M Tris-EDTA buffer (pH 9.0). The sections were heated to 125°C for 30 sec and then cooled to 90°C for 10 sec, the sections were then kept in the buffer until room temperature. The sections were incubated with

3% H₂O₂-methanol for 20 min and washed with phosphate-buffered saline (PBS, pH 7.4). After that the sections were incubated with rabbit anti-phospho-Ser536-p65 antibody (Abcam, MA, the antibody binds specifically to the Ser536-phosphorylated form of p65 and does not cross-react with non-phosphorylated p65 or any other members of the NF-κB family) at 20 mg/ml in antibody diluent (Dako, Carpinteria, CA) overnight, followed by rinsing with PBS. Subsequently, the sections were incubated with a goat anti-rabbit secondary antibody coupled with peroxidase provided by the Dako ChemMate EnVision Detection Kit (Dako) for 25 min, and washed with PBS. The peroxidase reaction, using 3,3'-diaminobenzidine tetrahydrochloride (Dako), was performed for 8 min. Sections known to stain positively were included as positive controls. The negative controls used PBS instead of the primary antibody. In all staining procedures, the positive controls showed clear staining, and there was no staining in the negative controls.

The sections were microscopically examined and scored independently by Lewander A and Gao J without any information on the clinicopathological data. The slides were initially classified, in a subjective matter, as negative (<5% of positive cells), weak, moderate and strong staining in the cytoplasm of normal epithelial cells, and tumor cells located in the entire area or invasive margin of primary tumor and metastasis, irrespective of the percentage of positive cells. For statistical analysis, we combined the negative cases with weak-stained cases as a group called weak expression, and combined moderate- and strong-stained cases as a group called strong expression, based on the similarities of the clinicopathological features. The expression in the nucleus of normal epithelial and tumor cells

was classified as negative (<5% of the nucleus stained) or positive expression. To avoid artificial effects, cells in areas with necrosis, with poor morphology, or in the margins of sections were excluded from the analysis.

Statistical analysis

The significance of the difference in phospho-Ser536-p65 expression between normal mucosa, primary tumor and metastasis was tested by using McNemar methods. The relationships between phospho-Ser536-p65 expression and clinicopathological variables were tested by Pearson Chi-square method, and the relationships to survival were examined by using Wald Cox's proportional hazard model. Survival curves were calculated by using the Kaplan-Meier method. In the survival analyses, only patients who died from colorectal cancer were classified as events, while those who died from other causes or were still alive were considered as censored. Two-sided p values of <0.05 were considered statistically significant.

Results

Phospho-Ser536-p65 expression in the cytoplasm of normal mucosa, primary tumor and metastasis in the lymph node

Table 1 presents the frequency of phospho-Ser536-p65 cytoplasmic expression in normal mucosa, entire primary tumor, invasive margin of primary tumor (excluding 27 cases that did not have visible invasive margin), and metastasis in the lymph node. We compared the staining intensity of phospho-Ser536-p65 in normal mucosa with entire primary tumor in the 128 matched cases by using the McNemar test. There were significantly more samples having strong expression among the primary tumors compared to normal mucosa ($p < 0.0001$), i.e., among the 128 cases, 56 cases had weak expression in normal tissue but strong expression in tumor, in contrast, only 5 cases had strong expression in normal tissue, but weak expression in tumor, the rest (67 cases) had identical expression in both normal and tumor tissue. There was no significant difference in the frequency of the expression between entire primary tumors and metastasis in the 12 matched cases ($p = 0.22$).

We also compared the staining intensity of phospho-Ser536-p65 in normal mucosa with that in invasive margin of primary tumor by using the McNemar test, the results were similar to a comparison of the normal mucosa with entire primary tumor, i.e., the frequency of the strong staining was higher in the invasive margin of primary tumor than in normal mucosa ($p < 0.0001$).

Figure 1 shows phospho-Ser536-p65 expression in normal mucosa, primary tumor and metastases in the lymph node. There was no phospho-Ser536-p65 expression in normal mucosa but strong expression in the cytoplasm of primary and metastatic tumors.

Table 1 Phospho-Ser536-p65 expression in the cytoplasm of normal mucosa, primary tumour, and metastasis in the lymph node.

	Phospho-Ser536-p65 expression	
	Weak (%)	Strong (%)
Normal mucosa (n=156)	107 (69)	49 (31)
Primary tumour		
Entire tumour (n=203)	73 (36)	130 (64)
Invasive margin (n=176*)	60 (34)	116 (66)
Metastases (n=18)	10 (56)	8 (44)

* Excluding 27 cases that did not have a visible invasive margin.

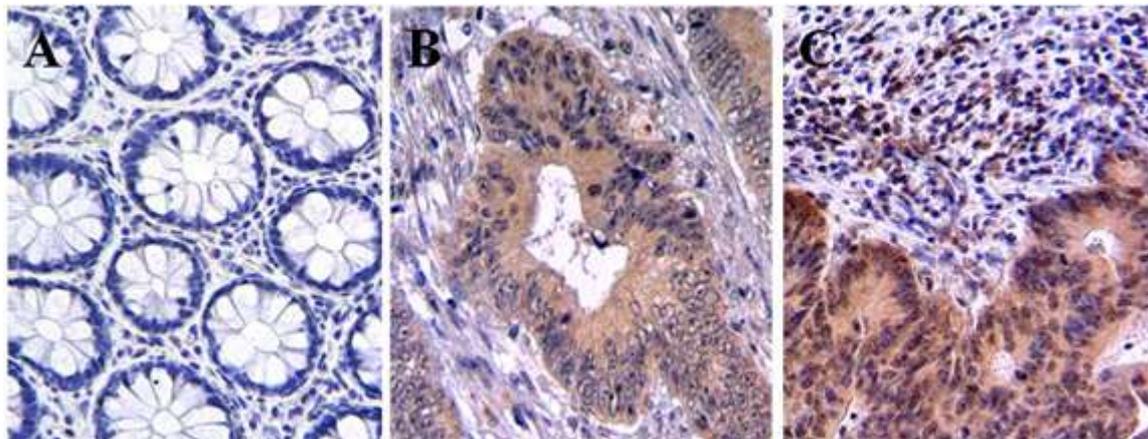


Fig. 1 - Phospho-Ser536-p65 expression was negative in normal mucosa (A), strong in primary tumor (B) and strong in metastases in the lymph node (C).

Phospho-Ser536-p65 expression in the cytoplasm in relation to clinicopathological variables

We first analyzed the relationship of cytoplasmic phospho-Ser536-p65 expression in the entire area of the primary tumor with survival. Univariate survival analysis showed that the strong expression of the phospho-Ser536-p65 correlated with worse survival of the patients ($p=0.02$, Figure 2). In the further multivariate analysis, the prognostic significance still remained after adjusting for patients' gender, age, tumor location, stage and differentiation ($p=0.04$, hazard ratio 1.89, 95% CI 1.03-3.47, Table 2). However, we did not find any statistically significant relationship of the phospho-Ser536-p65 expression with gender, age, tumor location, stage or differentiation by using Pearson Chi-square method ($p>0.05$, data not shown).

Then we analyzed the relationship of cytoplasmic phospho-Ser536-p65 expression in the invasive area of the primary tumor with survival. In the multivariate analysis, strong expression of the phospho-Ser536-p65 in the invasive margin of tumors was also related to worse survival independently of tumor stage ($p=0.04$, data not shown). However the prognostic significance was lost when we included patients' gender, age, tumor location and differentiation ($p=0.13$, by Wald multivariate Cox regression analysis, data not shown).

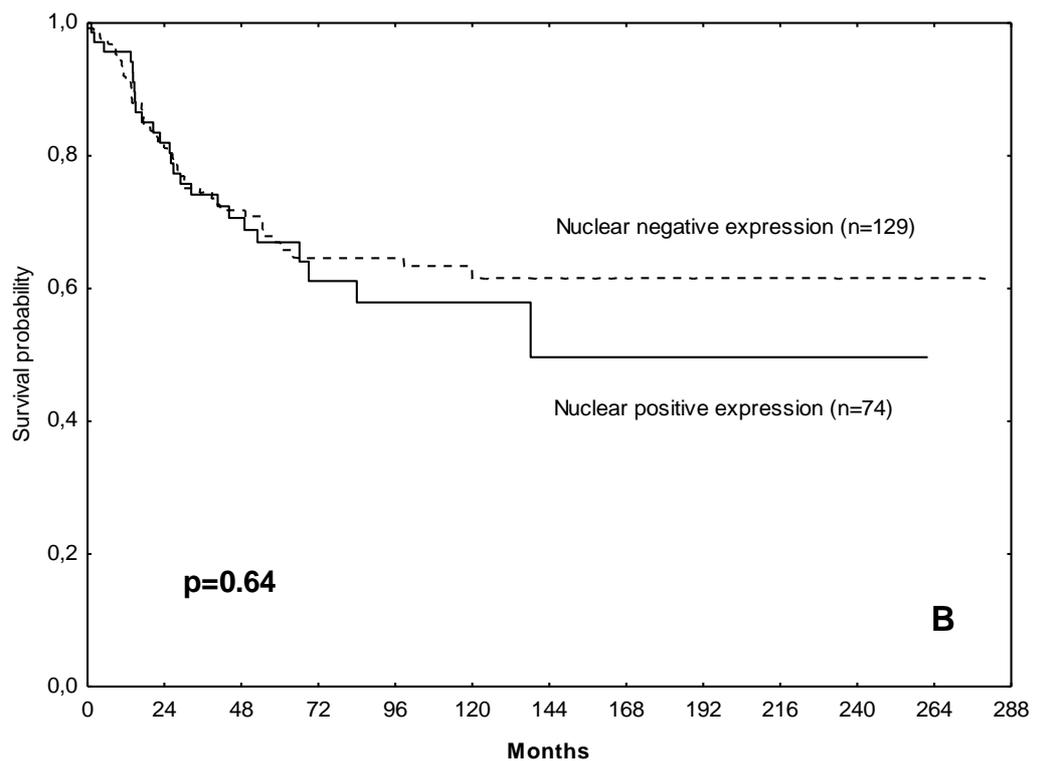
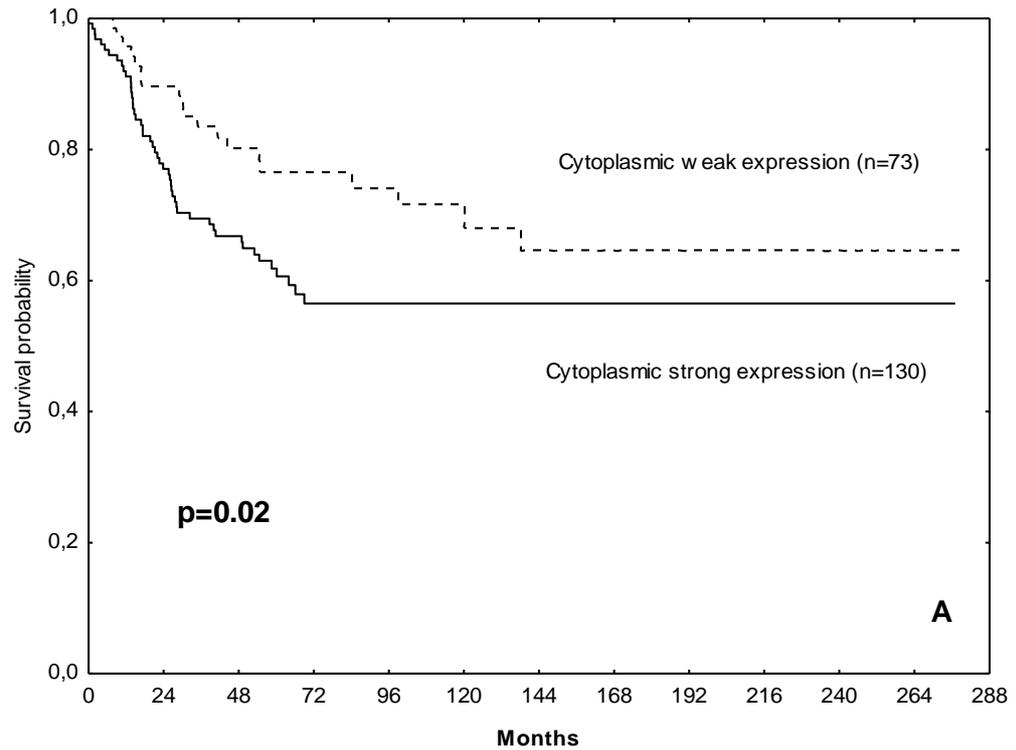


Fig. 2 - Phospho-Ser536-p65 expression in the cytoplasm (A) or in the nucleus (B) of the primary tumor in relation to survival in the patients with colorectal cancer.

Table 2 Multivariate Cox regression analysis of phospho-Ser536-p65 expression in the cytoplasm, gender, age, tumour location, stage and differentiation to survival in colorectal cancer

Variable	No of patients	Cancer death Hazard ratio	95% CI	p-value*
Phospho-Ser536-p65				0.04
Weak	72	1.00		
Strong	123	1.89	1.03-3.47	
Gender				0.54
Male	104	1.00		
Female	91	1.18	0.70-1.97	
Age (years)				0.14
<70	74	1.00		
≥70	121	1.52	0.87-2.68	
Tumour location				0.14
Colon	98	1.00		
Rectum	97	0.67	0.39-1.14	
Stage				<0.0001
I	26	1.00		
II	83	1.93	0.42-8.80	
III	53	6.03	1.35-26.91	
IV	33	38.3	8.77-167.91	
Differentiation				0.53
Better	138	1.00		
Poorer	57	1.18	0.70-1.99	

* by Wald multivariate Cox regression analysis

Expression of phospho-Ser536-p65 in the nucleus

There was a clear positive relationship between phospho-Ser536-p65 expression in the cytoplasm and that in the nucleus ($p < 0.001$, by using Pearson Chi-square test, Table 3).

There was no statistical difference in the frequency of phospho-Ser536-p65 expression in the nuclei from normal mucosa to primary tumors ($p = 0.20$, by McNemar test), and from primary tumor to metastasis in the lymph node ($p = 0.29$, by McNemar test), neither with survival by using Wald Cox's proportional hazard model ($p = 0.64$, Figure 2B) nor other clinicopathological variables by using Pearson Chi-square method ($p > 0.05$, data not shown).

Discussion

NF- κ B regulates the transcription of many genes which are responsible for immune response, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis [2,11]. Abnormalities in NF- κ B regulation are involved in multiple human pathologies including tumors. The function of NF- κ B is inhibited by binding to nuclear factor- κ B inhibitor (I κ B) and the release of activated NF- κ B follows proteasome-mediated degradation of I κ B resulting from phosphorylation of the inhibitor and finally conjugation with ubiquitin [2,10,11]. Since NF- κ B is responsible for the regulation of many other genes, changed expression of the NF- κ B and I κ B proteins could be potentially involved in tumor development. In the present study, we found that expression of NF- κ B p65 phosphorylated at Serine-536 was increased in primary tumor compared to normal mucosa. However, the frequency of the protein expression in the metastases in lymph nodes was not different from primary tumor. Unregulated NF- κ B in primary tumor compared with normal tissue has been found in previous studies. Lind et al [13] used an electrophoretic mobility shift assay technique and demonstrated that NF- κ B in primary tumor was greatly increased compared with adjacent normal tissue from the same patients. Yu et al [14] examined the expression of NF- κ B p65 by using a monoclonal antibody against NF- κ B p65 in normal colorectal mucosa, colorectal adenomas and colorectal adenocarcinomas, and showed that NF- κ B p65 expression was significantly increased from normal mucosa to adenoma and to adenocarcinoma, furthermore the expression was increased with the transition from low to moderate and to high dysplasia of adenoma.

Taken together, these results indicate that the NF- κ B p65 may play a role in earlier development of colorectal cancer.

The most interesting finding in the present study, also the first report, is that the expression of phospho-Ser536-p65 in the cytoplasm was significantly correlated to survival in the patients with colorectal cancer. The strong expression predicted worse survival of the patients independently of patients' gender, age, tumor location, stage and differentiation. Even though the p value is marginal ($p=0.04$) hazard ratio 1.89 (95% CI 1.03-3.47) is nearly two-times higher when comparing the strong expression with the weak-expression group.

Unexpectedly, the NF- κ B p65 in the nucleus did not give clinicopathological significance. Several other studies in colorectal and pancreatic cancers also showed cytoplasmic expression of NF- κ B p65, and further experiments demonstrated that the majority of NF- κ B p65 which is freed from I κ B remained in the cytoplasm [14-16]. During activation, NF- κ B p65 is phosphorylated at several residues. What residues, when and where seem to be dependent on different stimuli that activate NF- κ B [4]. There was a clear positive relationship between phospho-Ser536-p65 expression in the cytoplasm and that in the nucleus in the present study ($p<0.001$, Table 3), it may suggest that when the phosphorylated cytoplasmic NF- κ B is accumulated to a certain level, it will transfer to the nucleus. We did not see a direct relationship between nuclear NF- κ B and survival, which may depend on a matter of time point and/or the number of the cases etc. In fact, in the survival curves of the nuclear NF- κ B we saw a trend of the positive NF κ B expression resulting in worse survival (similar to the cytoplasmic expression in relation to

Table 3 Relationship between cytoplasm and nuclear staining in primary tumour.

Cytoplasmic expression	Nuclear expression		p-value*
	Negative (%)	Positive (%)	
Weak (n=73)	58 (79)	15 (21)	<0.0001
Strong (n=130)	71 (55)	59 (45)	

* by Pearson Chi-square test,

survival). Sakurai et al. [6] was first to report that upon tumor necrosis factor- α induced activation, I κ B kinase phosphorylates NF- κ B p65 at Serine-536 and that this occurs in the cytoplasm of HeLa cells before activated p65 translocates into the nucleus. Our present result showing the cytoplasmic staining by the antibody against phospho-Ser536-p65 further confirmed that phosphorylated NF- κ B p65 was in the cytoplasm of colorectal cancers, as well as in the nucleus. It has been shown that even under long-term stimulation, only up to 20% of NF- κ B p65 moved to the nucleus and the rest of the NF- κ B p65 still remained in the cytoplasm [16]. Possible explanations for this phenomenon are 1) I κ B is mutated and lost its binding to NF- κ B p65, and masks the nuclear translocation signal in NF- κ B p65, and 2) NF- κ B p65 is mutated and inhibited its binding to I κ B. Either mutation in I κ B or NF- κ B p65 prohibits their interaction, resulting in the changed activation and

mislocalisation of NF- κ B p65. We did not exclude a possibility that other biological factors which are related to cytoplasmic NF- κ B p65 play a role (or more important role) in colorectal cancer development/survival, rather than the cytoplasmic NF- κ B p65 itself, i.e., the cytoplasmic NF- κ B p65 may be a secondary event (as a bystander effect) due to co-activation of other biological factors.

In summary, we found increased levels of NF κ B p65 phosphorylated at Serine-536 in the cytoplasm of primary tumours compared with normal mucosa in colorectal cancer patients, however, we did not see a further increase from primary tumours to metastases in the lymph nodes, indicating that up-regulation of this protein is an early event in the development of colorectal cancer. Further, the cytoplasmic expression of NF- κ B p65 phosphorylated at Serine-536 predicts a worse prognosis independent of patients' gender, age, tumour location, stage and differentiation.

Conflicts of interest

None declared.

Acknowledgements

The authors thank Helen Richard, Cecilia Bergenwald, Gertrud Stridh, Gunnel Lindell and Kerstin Ingels, Department of Pathology, Linköping Hospital, Sweden, for kindly preparing tissue sections, and Dr. David Hinselwood (a Scottish doctor) at the Division of Oncology, Linköping University, Sweden, for revising this paper.

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