

# Neurofibromin Expression is Associated with Aggressive Disease and Poor Outcome in Colorectal Carcinoma

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**Abstract.** *Aim: To assess the predictive and prognostic value of neurofibromin (NF) expression in colorectal carcinoma (CRC). Materials and Methods: The present series consists of archival samples from 191 patients with stage I, II, III, or IV CRC treated between 1981 and 1990 at the Turku University Hospital (Finland). Tumor biopsies as microarray blocks were analyzed for expression of NF by immunohistochemistry. Different grading systems were tested for NF expression. Results: A significant correlation between NF expression and tumor localization was found, with tumors arising in the colon showing intense NF expression more often than those arising in the rectum ( $p=0.014$ ). Higher expression of NF was more common in tumors not responding to treatment ( $p=0.004$ ). Tumors with multiple metastases showed higher expression of NF than those with single metastasis only ( $p=0.025$ ). Furthermore, NF expression showed a borderline ( $p=0.068$ ) correlation with gender; tumors of women showed higher NF expression than those of males. On the other hand, NF expression was not significantly associated with tumor recurrence, age, lymph node involvement, tumor grade and tumor stage or disease outcome. Conclusion: Positive NF expression in CRC is a sign of aggressive disease and poor outcome.*

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Colorectal cancer (CRC) is the third most common cancer in the world, also ranking as the third most common cause of cancer-related deaths (1). Despite the fact that most CRC patients undergo potentially curative surgery and adjuvant chemotherapy administered, approximately 50% of all patients initially considered curable by surgery, will subsequently relapse and die of their disease (2).

The etiology of CRC remains open and the overall outcome is poor (3). However, some fundamental advances in our understanding of the biology and genetics of CRC have been made recently (4-5).

CRC arises from progressive accumulation of genetic and epigenetic changes (*e.g.* chromosomal instability, microsatellite instability and methylation pathway that cause the transformation of normal colon mucosa cells into adenocarcinoma (6, 7).

Alterations in *NF1* gene mutations and neurofibromin (NF) expression have been found in colon adenocarcinoma, myelodysplastic syndrome, anaplastic astrocytoma and small-cell lung carcinoma (8-9). NF, the protein encoded by *NF1* gene, is a cytoplasmic protein predominantly expressed in neurons, Schwann cells, oligodendrocytes and leucocytes (10). It functions as a GTPase, activating protein (GAP) for Ras by accelerating the conversion of active Ras-GTP to inactive Ras-GDP (11, 12). NF acts as a negative regulator of the Ras/mitogen-activated protein kinase signaling pathway *via* suppression of active Ras (13). Therefore, the negative regulation of Ras activity classifies *NF1* gene as a tumor suppressor gene.

To date, little is known about the role of tumor suppressor NF in CRC, and data on its potential prognostic value in these tumors are lacking. In this study, we examined the expression of NF in 191 CRCs using immuno-histochemistry

(IHC) and correlated the results with the established clinicopathological variables and outcome of the disease.

**Materials and Methods**

The present series consists of tissue samples obtained from 191 patients with stage I, II, III, or IV CRC who underwent bowel resection during 1981 and 1990 at Turku University Hospital (TUH, Finland), available for study at the archives of the Department of Pathology. IHC staining was performed at the Department of Pathology, Benghazi University, Benghazi, Libya. All pertinent clinical and histopathological data of the patients were collected from the patients' case records as summarized in Table I. All patients have been prospectively followed-up until death or when last seen alive on their clinical visit (March 2007), with a median follow-up time of 77 months (range=2-263). The study was approved by the TUH Ethics Committee and conducted in accordance with the endorsement of the National Authority for Medico-legal Affairs

*Tissue microarray.* Archival paraffin-embedded samples were used to build up tissue microarray (TMA) blocks for IHC staining. Areas of invasive tumor with the lowest degree of differentiation, abundant in cells with the highest number of mitoses, were chosen from the original blocks. Necrotic and autolytic areas and areas containing predominantly stromal tissue were excluded. For tumors producing abundant intra- or extracellular mucin, invasive areas with the highest number of epithelial cells were chosen. These representative areas were marked by an experienced pathologist on hematoxylin and eosin (HE)-stained slides from selected paraffin blocks and a cylinder of tissue (1 mm in diameter) was cut with a TMA instrument (Beecher Instruments, Sun Prairie, WI, USA) into a new paraffin block. This size of tissue section (1-mm wide) was equal to the often used three cores, 0.6-mm wide (20-23). As the core was larger than usual, sampling differences were less in 0.6-mm cores. Serial 4-µm sections were then cut from the TMA paraffin blocks. The sections were mounted on ChemMate™ Capillary Gap plus Slides (gray) (Dako, Glostrup, Denmark). Normal colorectal mucosa was selected at least 2 mm apart from the malignant tissues of the section. If available, another normal sample was obtained from normal colorectal mucosa at either of the resection margins in the surgical specimens. On average, two normal controls were available. Lymphatic follicles and hyperplastic, as well as inflamed areas were avoided. To obtain enough mucosa for tissue array, all tangentially cut areas were avoided.

*Neurofibromin (NF) immunostaining.* Immunohistochemical (IHC) analysis was done using an automated system (BenchMark XT; Ventana Medical System, Inc., Tucson, AZ, USA). This fully automated processing of code-labeled slides included baking of the slides, solvent-free deparaffinization, antigen retrieval in the cell conditioning buffer CC1 (Mild: 36 minutes conditioning and standard: 60 minutes conditioning), incubation with rabbit monoclonal anti-neurofibromin antibody, 2.0 ml ready-to-use from Spring Bioscience at a dilution of 1:50 for 32 minutes, at 37°C (clone: NF n27b, Catalog No: M3794; Parkway, CA, USA), as well as application of ultra view™ universal DAB inhibitor, ultraview universal DAB chromogen, ultraview universal DAB H<sub>2</sub>O<sub>2</sub>, ultraview universal DAB copper and ultraview universal HRP multimer.

Table I. Clinicopathological characteristics of patients.

Characteristic	Number of patients (%)
Gender	
Male	93 (48.7%)
Female	98 (51.3%)
Age (years)	
<65 years	87 (45.5%)
>65 years	104 (54.5%)
Primary tumor status	
T1	5 (2.6%)
T2	14 (7.3%)
T3	114 (59.7%)
T4	58 (30.4%)
LN involvement	
No	122 (63.9%)
Yes	69 (36.1%)
Stage	
I	19 (10%)
II	103 (54%)
III	30 (15.6%)
IV	39 (20.4%)
Histological grade	
Grade I	27 (14.1%)
Grade II	140 (73.3%)
Grade III	24 (12.6%)
Localization	
Right colon	65 (34%)
Left colon	69 (36%)
Rectum	57 (30%)
Distant Metastasis	
No	152 (79.6%)
Yes	39 (20.4%)
Recurrence during the follow-up	
Yes	56 (29.3%)
No	96 (50.3%)
Unknown	39 (20.4%)
Status at the end of follow-up	
Alive	50 (26.2%)
Dead as result of disease	87 (54.5%)
Dead from other cause (s)	54 (28.3%)
Response to therapy	
Response	9 (4.7%)
No response	28 (14.7%)
Unknown	154 (80.6%)

Counterstaining with hematoxylin II (C00758) was performed for 4 min followed by post-counterstaining with blueing reagent (B11129) for 4 min. After staining, the sections were dehydrated in ethanol, cleared in xylene and covered with Mountex and coverslips.

*Evaluation of NF staining.* NF staining was evaluated using regular light microscope at the magnification of x40, blinded by the information on tumor grade, stage or clinical outcome. Membranous and cytoplasmic staining were evaluated separately. Two different grading (A and B) systems were applied to assess the patterns of NF expression in tumor cells. In system A, the staining was graded

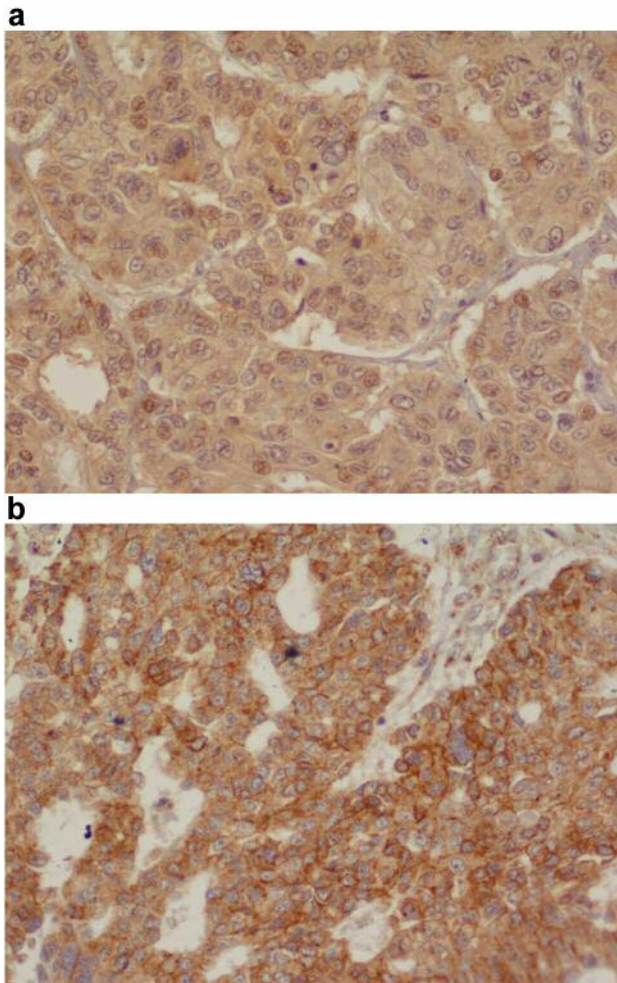


Figure 1. *a*: Weak neurofibromin expression in CRC ( $\times 40$  magnification). *b*: Moderate and strong neurofibromin expression in CRC ( $\times 40$  magnification).

into four categories: 0, no expression (no detectable staining); 1, weak staining; 2, moderate staining; and 3, strong staining intensity. In system B, staining was graded in two categories: 1, no/weak expression; and 2, moderate/strong expression. Both systems were statistically tested.

In calculating the staining indexes, cytoplasmic and membranous index, the intensity of staining and the fraction of positively stained cells were taken into account using the following formula:

$$I = 0 \times f_0 + 1 \times f_1 + 2 \times f_2 + 3 \times f_3$$

Where "I" is the staining index and  $f_0$ - $f_3$  are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and 3 (14, 15).

**Statistical analysis.** SPSS for Windows SPSS 19.0.1 (IBM, Armonk, NY, USA) was used for statistical analysis. Frequency tables were analyzed using the Chi-square test, with Fisher's

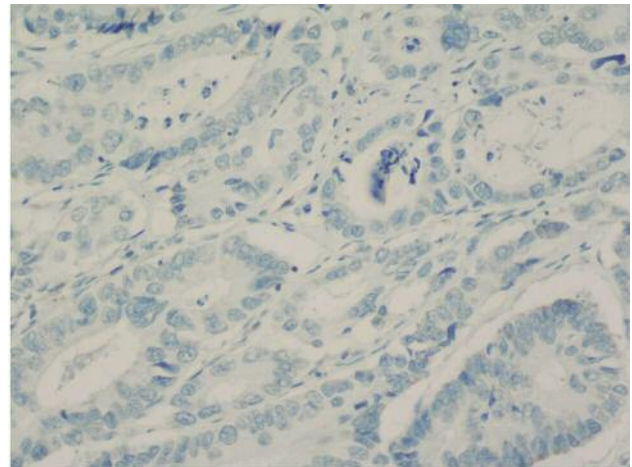


Figure 2. Negative neurofibromin expression in CRC ( $\times 40$  magnification).

exact test (where appropriate), or likelihood ratio (LR) statistics to assess the significance between categorical variables. Differences in the means of continuous variables were analyzed using analysis of variance ANOVA or non-parametric tests (Mann-Whitney, Kruskal-Wallis) tests. Reported  $p$ -values are from two-sided tests; in all analyses,  $p < 0.05$  was regarded as statistically significant.

## Results

**Description of NF expression patterns.** The expression pattern of NF was cytoplasmic and membranous in the tumor area. Examples of the staining patterns of NF are illustrated in Figures 1a, 1b and 2. Of the 191 tumors, 176 (92.1%) were considered positive (staining intensity +1, +2, +3; Figures 1a, b), whereas 15 (7.9%) were considered negative (staining intensity 0; Figure 2).

**NF expression correlates with clinicopathological features.** A significant correlation between NF expression and tumor localization was found, tumors arising in the colon showed intense NF expression more often than tumors arising in the rectum ( $p = 0.014$ ).

NF expression with higher intensity was more common in tumors not responding to treatment ( $p = 0.004$ ). Also tumors with multiple metastases expressed NF more intensely than tumors with single metastasis only ( $p = 0.025$ ). Furthermore, NF expression showed a borderline ( $p = 0.068$ ) correlation with gender, with tumors of the women showing higher NF expression than tumors of men. On the other hand, NF expression was not significantly associated with tumor recurrence, age, lymph node involvement, tumor grade and tumor stage or disease outcome at study endpoint (Table II).

Table II. Correlation between neurofibromin expression and clinicopathological features of colorectal carcinoma.

Features	Number of cases (%)	Neurofibromin expression				p-Value
		Negative	Weak	Moderate	Strong	
Gender						0.195
Male	93(48.7%)	7(7.5%)	26(28%)	34(36.6%)	26(28%)	
Female	98(51.3%)	8(8.2%)	15(15.3%)	45(45.9%)	30(30.6%)	
Age group (years)						0.229
<65	87(45.5%)	8(9.2%)	15(17.2%)	33(37.9%)	31(35.6%)	
>65	104(54.5%)	7(6.7%)	26(25.0%)	46(44.2%)	25(24.0%)	
Primary tumor status						0.204
T1	5(2.6%)	0(0%)	0(0%)	2(40%)	3(60%)	
T2	14(7.3%)	1(7.1%)	5(35.7%)	4(28.6%)	4(28.6%)	
T3	114(59.7%)	7(6.1%)	27(23.7%)	53(46.5%)	27(23.7%)	
T4	58(30.4%)	7(12.1%)	9(15.5%)	20(34.5%)	22(37.9%)	
LN involvement						0.299
No	122(63.9%)	10(8.2%)	25(20.5%)	56(45.9%)	31(25.4%)	
Yes	69(36.1%)	5(7.2%)	16(23.2%)	23(33.3%)	25(36.2%)	
Distant metastasis						0.190
No	152(79.6%)	12(7.9%)	34(22.4%)	67(44.1%)	39(25.7%)	
Yes	39(20.4%)	3(7.7%)	7(17.9%)	12(30.8%)	17(43.6%)	
Stage						0.406
I	19(10%)	1(5.3%)	5(26.3%)	6(31.6%)	7(36.8%)	
II	103(54%)	9(8.7%)	20(19.4%)	50(48.5%)	24(23.3%)	
III	30(15.6%)	2(6.7%)	9(30%)	11(36.7%)	8(26.7%)	
IV	39(20.4%)	3(7.7%)	7(17.9%)	12(30.8%)	17(43.6%)	
Histological grade						0.895
Grade I	27(14.1%)	2(7.4%)	7(25.9%)	9(33.3%)	9(33.3%)	
Grade II	140(73.3%)	11(7.9%)	30(21.4%)	61(43.6%)	38(27.1%)	
Grade III	24(12.6%)	2(8.3%)	4(16.7%)	9(37.5%)	9(37.5%)	
Localization						0.014
Colon	134(70%)	12(9%)	23(17.2%)	64(47.8%)	35(26%)	
Rectum	57(30%)	3(5.3%)	18(31.6%)	15(26.3%)	21(36.8%)	
Recurrence						0.482
Yes	56(29.3%)	6(10.7%)	11(19.6%)	22(39.3%)	17(30.4%)	
No	96(50.3%)	6(6.3%)	23(24%)	45(46.9%)	22(22.9%)	
Unknown	39(20.4%)	No statistical data	No statistical data	No statistical data	No statistical data	
Status at the end of FU						0.937
Alive	50(26.2%)	4(8%)	10(20%)	21(42%)	15(30%)	
Dead as result of disease	87(54.5%)	8(9.2%)	15(17.2%)	35(40.2%)	29(33.3%)	
Dead from other causes	54(28.3%)	No statistical data	No statistical data	No statistical data	No statistical data	
Response to therapy						0.004
Response	9(4.7%)	4(44.4%)	2(22.2%)	2(22.2%)	1(11.2)	
No response	28(14.7%)	0(0%)	7(25%)	13(46.4%)	8(28.6%)	
Unknown	154(80.6%)	No statistical data	No statistical data	No statistical data	No statistical data	
Multiple Distant Metastases	12(31%)	0(0%)	1(8%)	5(42%)	6 (50%)	0.025

**Survival analysis.** In the Kaplan-Meier survival analysis, there was a difference in recurrence-free survival (RFS) or disease-free survival (DFS) between patients with tumors expressing no/weak NF and those with NF overexpression ( $p=0.149$ ) (Figure 3). Interestingly, at 3-year follow-up, 30% of the patients with tumors overexpressing NF showed recurrence as compared to only 15% of the patients whose tumors showed no/weak expression.

## Discussion

This study is a continuation of our efforts to further elucidate the biology of CRC and identify more effective prognostic factors. Colon cancer arises from progressive accumulation of genetic and epigenetic changes that causes the transformation of normal colon mucous cells into adenocarcinoma (6, 16). The role of *NFI* gene or its protein

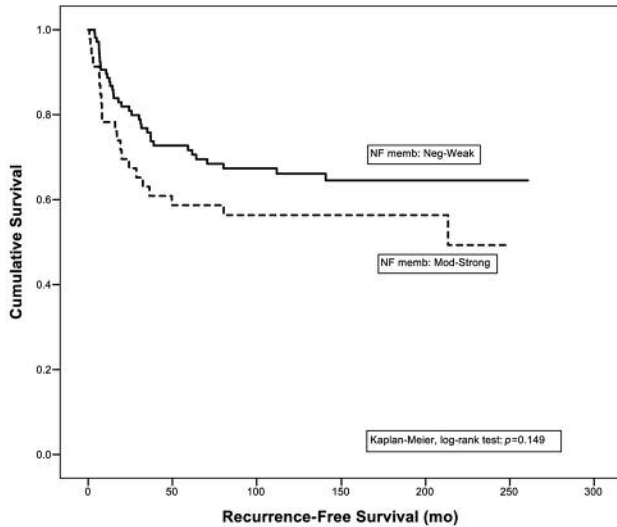


Figure 3. Neurofibromin expression (neg-weak vs. mod-strong) as determinant of disease-free survival in univariate (Kaplan-Meier) analysis ( $p=0.149$  log-rank).

NF in the development and progression of colon cancer is unknown. The aim of this study was to examine the role of tumor suppressor NF in CRC. We examined the expression of NF by IHC in 191 cases of CRC. NF expression was detected in 92.1% of CRC cases and found to correlate significantly with tumor localization, tumors not responding to treatment, tumors with multiple metastases and those (less significantly) to gender. In contrast, no correlation was established with tumor recurrence, age, lymph node involvement, tumor grade and stage.

Two different grading systems were applied to assess the patterns of neurofibromin expression. In system A, a 4-tier grading (of 0, 1, 2, 3;) (no, weak, moderate, strong staining intensity, respectively) was used, which was shown to correlate significantly with tumors' localization ( $p=0.014$ ), and tumors not responding to treatment ( $p=0.004$ ). On the other hand, this grading failed to correlate with tumor recurrence, age, gender, lymph node involvement, distant metastases, tumor grades and stages.

In system B, a 2-tier grading 0/1 vs. 2/3 (negative/weak vs. moderate/strong staining intensity) was used and shown to correlate significantly with failing treatment response ( $p=0.042$ ), multiple metastases ( $p=0.025$ ), and (borderline significantly) with gender. Similarly, no correlation was found with tumor localization, tumor recurrence, age, lymph node involvement, tumor grades and stages.

Interestingly, we observed a significant correlation between NF expression and tumor localization; positive expression of NF was significantly ( $p=0.014$ ) associated with left-sided

tumors, whereas NF expression was lost more frequently in right-sided colon cancers. This suggests that there may be differences between the normal right and left colonic segments that could favor malignant transformation through different molecular pathways. Such differences are probably related to different molecular profiles of tumors, microsatellite instability, and methylator phenotypes being associated with right-sided tumors, as well as chromosomal instability with left-sided tumors (17, 18). Future molecular studies are necessary to confirm this hypothesis.

In conclusion, the present work showed NF expression to be significantly associated ( $p=0.004$ ) with poor response to treatment and tumors with multiple metastases ( $p=0.025$ ), suggesting that positive NF expression in CRC might be an indicator of aggressive disease and poor outcome. However, larger series of CRCs need to be analyzed to validate these observations.

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