Expression of NDRG2 is down-regulated in high-risk adenomas and colorectal carcinoma

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Abstract

Background: It has recently been shown that NDRG2 mRNA is down-regulated or undetectable in several human cancers and cancer cell-lines. Although the function of NDRG2 is unknown, high NDRG2 expression correlates with improved prognosis in high-grade gliomas. The aim of this study has been to examine NDRG2 mRNA expression in colon cancer. By examining affected and normal tissue from individuals with colorectal adenomas and carcinomas, as well as in healthy individuals, we aim to determine whether and at which stages NDRG2 down-regulation occurs during colonic carcinogenesis.

Methods: Using quantitative RT-PCR, we have determined the mRNA levels for NDRG2 in low-risk (n = 15) and high-risk adenomas (n = 57), colorectal carcinomas (n = 50) and corresponding normal tissue, as well as control tissue from healthy individuals (n = 15). NDRG2 levels were normalised to β-actin.

Results: NDRG2 mRNA levels were lower in colorectal carcinomas compared to normal tissue from the control group (p < 0.001). When comparing adenomas/carcinomas with adjacent normal tissue from the same individual, NDRG2 expression levels were significantly reduced in both high-risk adenoma (p < 0.001) and in colorectal carcinoma (p < 0.001). There was a trend for NDRG2 levels to decrease with increasing Dukes’ stage (p < 0.05).

Conclusion: Our results demonstrate that expression of NDRG2 is down-regulated at a late stage during colorectal carcinogenesis. Future studies are needed to address whether NDRG2 down-regulation is a cause or consequence of the progression of colorectal adenomas to carcinoma.
Background

N-myc Downstream Regulated Gene 2 (NDRG2) is a member of a recently identified gene family which has been implicated in human nervous system disorders and cancer [1]. Although the four members of this family contain a putative α/β-hydrolase fold, it is unclear whether or not they have enzymatic activity [2]. NDRG1 was first identified as a gene under negative regulation by N-myc in early mouse development [3]. NDRG2 was identified through sequence homology and is implicated in cell growth, differentiation and neurodegeneration [4-7]. Recently, it has been shown that expression of NDRG2 is transcriptionally repressed by c-Myc [8].

Several studies have suggested that NDRG2 mRNA is down-regulated or undetectable in a number of human cancers and cancer cell-lines [4,9-11]. Semi-quantitative RT-PCR was used to demonstrate that NDRG2 expression levels were reduced in squamous cell carcinoma, pancreatic cancer and glioblastoma compared to normal tissue [4,9,11]. NDRG2 expression levels in gliomas and meningiomas were significantly attenuated in high-grade compared to low-grade tumors [4,10]. In meningiomas, higher expression of NDRG2 mRNA correlated with clinically less aggressive tumors [10]. Furthermore, NDRG2 was identified as a gene whose expression in high-grade gliomas was positively correlated with survival [12]. Forced NDRG2 overexpression in a human glioblastoma cell-line markedly inhibited cell proliferation [4]. These findings implicate NDRG2 as a possible tumor suppressor gene.

Prompted by the finding that NDRG2 expression correlates inversely with tumor grade in various cancers, we set out to analyse NDRG2 mRNA expression during colorectal carcinogenesis in humans.

Methods

Subject population

The KAM cohort (Kolorektal cancer, Av og Miljø) is based primarily on the screening group of the Norwegian Colorectal Cancer Prevention study (the NORCCAP study, ID number at Clinicaltrials.gov NCT00119912) in the county of Telemark, Norway [13,14]. Additionally, a series of colorectal cancer cases were recruited to the KAM cohort from routine clinical work at Telemark Hospital and Ulleval University Hospital in Oslo. A total of 20,780 men and women, age distribution 50–64 years, randomly drawn from the population registries in Oslo (urban) and the county of Telemark (mixed urban and rural) were invited to have a flexible sigmoidoscopy (FS) screening examination with or without (1:1) an additional faecal occult blood test (FOBT). A total of 777 (4%) individuals were excluded according to the exclusion criteria [13]. The KAM biobank currently consists of 234 colorectal cancer, 1044 adenoma (229 high-risk, 762 low-risk and 53 hyperplastic polyps) and 400 control specimens. Controls were defined as individuals with normal findings at FS. The KAM study is approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate. In the present study we have analyzed carcinomas (n = 50), adenomas (n = 72) and controls (n = 15). Each case was classified according to the degree of malignancy. A sample of control tissue was collected 30 cm from the anus of patients with adenomas, whereas two samples of control tissue were taken from the surgical specimen from patients with carcinomas: one sample in close proximity (normal adjacent) and one sample as far away from the tumor as possible (normal distant). Control samples were collected from individuals without adenomas or carcinomas. The histology of the adenomas was examined by two histopathologists independently. The degree of dysplasia was determined as either mild/moderate (n = 52) or severe (n = 20). The two pathologists reached the same conclusion in all cases. Furthermore, adenomas were classified as either low-risk (n = 15) or high-risk (n = 57). A high-risk adenoma is defined as an adenoma measuring ≥ 10 mm in diameter and/or with villous components and/or showing severe dysplasia [13]. The vast majority of CRC samples had 75–80% tumor cells surrounded by stroma, as evaluated by hematoxylin and eosin staining by a pathologist. The distribution of gender and age among controls and cases with colonic carcinoma or adenoma are shown in Table 1.

Table 1: Characteristics of cases and healthy persons in this study.

|                | Controls | Cases |                |                |
|----------------|----------|-------|----------------|----------------|
|                | Low-risk adenomas | High-risk adenomas | Carcinomas |
|                | n = 15 | n = 15 | n = 57 | n = 50 |
| Men           | 5      | 12     | 40      | 31   |
| Women         | 10     | 3      | 17      | 19   |
| Mean age (SD) | 57.3 (4.9) | 56.7 (4.4) | 56.4 (3.8) | 71.8 (10.5) |

1A high-risk adenoma is defined as an adenoma measuring ≥ 10 mm in diameter and/or with villous components and/or showing severe dysplasia.
2There are significant differences in age among the four groups of healthy and affected individuals at 95% confidence level (Kruskal-Wallis test).
Cancer Profiling Array (CPA)
The Cancer Profiling Array II (Clontech) was hybridised with 50 ng of radioactively labelled NDRG2 probe according to the manufacturer's instructions. The 460 bp NDRG2 probe was generated by PCR using the primers 5’CTCACCTGTGGAGACACCAT3’ and 5’GGGTGATATCACCTCCACGCAGT3’. The hybridised array was exposed to a phosphorimaging screen for 24 hours and the intensity of each spot was quantified using ImageQuant (Molecular Dynamics). The CPA consists of paired cDNA samples generated from the total RNA of normal and tumor tissue. Because the array is normalised for several housekeeping genes, quantification of the hybridisation signal provides an estimate of relative transcript abundance.

RT-PCR
Total RNA was purified from tissue as recommended by the manufacturers using an e.z.n.a. Gel Extraction kit (Omega Biotek). The tissue had been snap-frozen in liquid N2 and stored at -80°C before RNA purification. RNA purification included a DNase treatment. The cDNA synthesis was performed on approximately 200 ng RNA per 10 μl using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Nærum, Denmark). Quantitative RT-PCR was performed on an ABI7500 sequence detection system (Applied Biosystems) in Universal Mastermix (Applied Biosystems) using 220 nM probe and 700 nM primers for NDRG2. NDRG2 primers were NDRG2F: 5’CGATCCTTACCTACCAGATG3’ and NDRG2R: 5’CTCACTCTGAATCCACACGCT3’ and the probe was 5’FAM-CICACACTATAATCTTTGCCTCC-MGB-NFQ-3’.

Primers were designed using Primer Express v3.0 Software and obtained from DNA Technology A/S. Primers were designed within different exons and with a probe covering and obtained from DNA Technology A/S. Primers were designed using Primer Express v3.0 Software.

Results
Expression of NDRG2 mRNA in colonic adenomas and carcinomas
Preliminary experiments using a Cancer Profiling Array indicated that expression of NDRG2 mRNA was reduced in 9 out of 10 colonic tumors (Table 2). Using a two-tailed paired t-test, the decrease in NDRG2 expression in tumors as compared to the corresponding normal tissues was found to be statistically significant (p < 0.01). Prompted by this finding, we decided to analyse NDRG2 mRNA expression in normal and neoplastic tissue in a larger number of patients to confirm and extend our results.

Using real-time RT-PCR we have measured the levels of NDRG2 mRNA in colonic tissue from healthy individuals and from individuals with colorectal adenomas or carcinoma (Table 1). It was observed that the expression of NDRG2 mRNA in colorectal tissue is relatively low compared to the expression of β-actin, which was used for normalisation (Figure 1). Upon examination of the mean values in affected tissue (low- and high-risk adenomas and carcinoma), a trend towards a decreased NDRG2 expression with increasing tumor grade was observed (p < 0.001) (Figure 1).

Analysis of the data using a one-way ANOVA with Tukey's post test did not show any significant difference in NDRG2 mRNA level between the control group and either normal or affected tissue from individuals with adenoma (low- and high-risk). This was also the case when comparing normal tissue from individuals with colorectal cancer to the control group. However, when comparing affected tissue from individuals with colorectal cancer to corresponding tissue from a healthy control, a statistically sig-
NDRG2 mRNA levels are down-regulated during colorectal cancer carcinogenesis

Figure 1

NDRG2 mRNA levels are down-regulated during colorectal cancer carcinogenesis. mRNA expression of NDRG2 determined by real-time RT-PCR and normalised to β-actin in healthy individuals (Control), normal and affected tissue from the same individual with adenomas (low- or high-risk) and colorectal carcinoma. Normal (adjacent): normal sample close to the carcinoma, Normal (distant): normal sample far from the carcinoma. Each dot represents mean values of triplicate determinations. *** p < 0.001 compared to the control group using one-way ANOVA with a Tukey’s post test. A trend of decreased NDRG2 expression with increasing tumor grade was observed in affected tissue (p < 0.001).
significant difference in the level of NDRG2 mRNA was observed (p < 0.001) (Table 3).

Further analysis of the different groups of affected tissue using a paired two-tailed t-test showed that the level of NDRG2 in individuals with low-risk adenoma did not show any significant difference between normal and neoplastic tissue. However, a comparison of normal and high-risk adenoma from the same individual showed a highly statistically significant reduction (p < 0.001) in NDRG2 level. Finally, comparing the level of NDRG2 mRNA in normal tissue far (normal distant) and close (normal adjacent) to that of the tumor in the surgical specimens of CRC patients showed a statistically significant difference (p < 0.001) in both cases (Table 3).

Cases of adenomas can also be classified according to the diagnosed degree of dysplasia (mild/moderate or severe) (Table 4). When comparing affected tissue with normal tissue from the same individual, we found a statistically significant difference in NDRG2 expression for individuals with mild/moderate dysplasia (p < 0.001) (Table 4).

**Analysis of expression levels in carcinomas according to Dukes’ staging**

Colorectal cancer can be staged according to different systems. In this study all samples of colorectal carcinoma (CRC) were classified as Dukes’ stage A-C where C is the most advanced and metastatic stage. Figure 2 presents data showing that the level of NDRG2 mRNA decreases with increasing Dukes’ stage. By calculating linear regression on the data, the result was a statistically significant linear trend (p < 0.05) for decreasing NDRG2 levels with increasing tumor stage.

**Expression patterns of NDRG2 between genders in colorectal cancer**

The incidence of new cases of colorectal cancer in Norway is in the ratio 1:1 between the two genders [16]. Dividing all data collected in this study into groups of males and females showed a general lower level of NDRG2 expression in females with colorectal carcinoma (both normal and cancer tissue) (Figure 3). However, this difference was not statistically significant using a one-way ANOVA with Tukey’s post test.

**Discussion**

In the present study we demonstrated that NDRG2 mRNA expression levels were lower in colonic tumors than in normal colon tissue from the same individual. This was observed using two distinct subject populations, one of which was a Norwegian cohort, the other a group of affected individuals based on a commercially available product. The difference in mRNA level is likely to be reflected at the level of NDRG2 protein, since NDRG2 mRNA levels have previously been shown to correlate well with protein levels [8].

In the Norwegian cohort, NDRG2 mRNA levels were statistically significantly reduced in colorectal carcinoma when compared to the healthy controls. In order to examine whether the risk of carcinoma is affected by changes in the microenvironment, expression levels of NDRG2 in the lesion were compared to normal adjacent tissue as well as to normal tissue distant from the tumor. NDRG2 mRNA was statistically significantly reduced in tumor compared to either normal tissue sample. No difference was observed between the adjacent and distant samples, suggesting that changes in NDRG2 expression in the carcinoma are not attributable to the microenvironment.

Recent studies have demonstrated that colorectal cancer is a heterogeneous disease with distinct molecular components. Distinct genetic or epigenetic alterations have been identified which correlate with the location of the tumors [17]. Although it was not investigated in this study, it could be interesting to compare NDRG2 expression in tumors located in either the proximal or distal colon.

There was a tendency for decreasing NDRG2 mRNA levels with increasing tumor stage according to Dukes’ staging of the CRC samples, and this trend was found to be signifi-

| Table 3: Mean values of normalised levels of NDRG2 mRNA in normal and affected colonic tissues. |
|-----------------------------------------------|
| mRNA level in normal | p<sub>a</sub> | mRNA level in adenomas/carcinomas | p<sub>b</sub> | p<sub>c</sub> |
| tissue Mean (SD) | Mean (SD) | |
| Control | 0.034 (0.009) | 0.0407 (0.017) | NS | NS |
| Low-risk Adenoma | 0.044 (0.014) | 0.0307 (0.011) | NS | NS |
| High-risk Adenoma | 0.041 (0.012) | 0.0136 (0.012) | NS | < 0.001 |
| Carcinoma Normal (distant) | 0.027 (0.015) | < 0.001 | < 0.001<sup>c</sup> |
| Carcinoma Normal (adjacent) | 0.034 (0.021) | < 0.001<sup>d</sup> | |

NS = not significant.

<sup>a</sup>p value when expression levels were compared to the control group of healthy individuals using one-way ANOVA with a Tukey’s post test.

<sup>b</sup>p value for comparison of mRNA expression levels in normal and tumor samples from the same individual using a paired two-tailed T-test.

<sup>c</sup>p value when normal (distant) and corresponding tumor sample were compared.

<sup>d</sup>p value when normal (adjacent) and corresponding tumor sample were compared.
significant using linear regression ($p < 0.05$). Our results are in agreement with that observed for other cancer types where NDRG2 expression is reduced in high-grade compared to low-grade tumors [4,10]. This trend indicates either that the loss of NDRG2 promotes tumor progression or that NDRG2 is inactivated by factor(s) present at advanced tumor stages. NDRG2 has previously been shown to be negatively regulated by the c-Myc oncoprotein [8] and it is possible that elevated levels of c-Myc would result in reduced expression of NDRG2. Thus, it could be interesting to elucidate whether or not an increased level of c-Myc, which is a frequent event in colorectal cancer [18], correlates with a decreased level of NDRG2. Measurement of c-Myc levels was not included in these studies. However, we have investigated a subset of the CRC samples ($n = 54$) from the KAM study for β-catenin expression by immunohistochemistry. All of the tested CRC samples are positive for cytoplasmic β-catenin and 72% are β-catenin positive in all nuclei (data not shown). The remaining samples contain nuclear β-catenin in occasional nuclei. This suggests that c-Myc levels are likely to be elevated since c-Myc is known to be positively regulated by nuclear β-catenin [19].

In order to determine the stage at which NDRG2 expression is down-regulated in the adenoma-carcinoma sequence we also examined normal and affected tissue

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Table 4: Mean values of normalised levels of NDRG2 mRNA in adenomas.

|                          | mRNA level in normal tissue Mean (SD) | p$^a$ | mRNA level in adenomas Mean (SD) | p$^a$ | p$^b$ |
|--------------------------|--------------------------------------|-------|----------------------------------|-------|-------|
| Mild/moderate Dysplasia  | 0.042 (0.012)                        | NS    | 0.032 (0.014)                    | NS    | < 0.001 |
| Severe Dysplasia         | 0.040 (0.014)                        | NS    | 0.034 (0.011)                    | NS    | NS     |

NS = not significant.

$^1$Cases of adenomas were divided into mild/moderate ($n = 52$) or severe ($n = 20$) according to the diagnosed degree of dysplasia.

$^a$p value when expression levels were compared to the control group of healthy individuals using one-way ANOVA with a Tukey’s post test.

$^b$p value for comparison of mRNA expression levels in normal and tumor samples from the same individual using a paired two-tailed T-test.
from low- and high-risk adenomas. When comparing affected tissue with normal tissue from the same individual, we found a statistically significant difference for individuals with high-risk adenomas. However, when compared to the control group of healthy individuals, only the affected tissue from individuals with colorectal carcinoma shows a statistically significant reduction in NDRG2 mRNA levels. Our results suggest that down-regulation of NDRG2 expression occurs during the progression from adenoma to carcinoma.

Whether down-regulation of NDRG2 in colorectal carcinoma is a cause or a consequence of malignant progression is at present unclear. Although the structure of NDRG2 resembles that of a hydrolase [2], its ability to function as an enzyme is presently unknown. It has recently been shown that overexpression of NDRG2 in a glioblastoma cell-line inhibits cell proliferation [4] and that NDRG2 expression correlates positively with survival in high-grade glioma [12]. NDRG2 levels are also reduced in several cancer types and cell-lines [4,9-11]. Thus, NDRG2 may have a general function in diverse tissues as a tumor suppressor gene. Future studies will be needed to examine whether increased NDRG2 levels in colorectal carcinoma correlate with improved prognosis.

**Conclusion**

In conclusion, NDRG2 mRNA levels were decreased in both high-risk colorectal adenoma and in colorectal carcinoma compared to corresponding normal colonic mucosa from the same individual. Furthermore, NDRG2 expression was reduced in colorectal carcinoma compared to normal tissue from healthy individuals. Our results suggest that NDRG2 down-regulation correlates with the progression of dysplastic tissue to carcinoma. Future studies are needed to address whether NDRG2 down-regulation is a cause or consequence of colorectal carcinogenesis.

**Competing interests**
The author(s) declare that they have no competing interests.

**Authors’ contributions**

LKV and CM conceived the idea of the study and designed the primers and probes. EHKK designed and administered the KAM study and collected the samples. LKV, MS and CFS extracted the RNA and carried out the cDNA synthesis. SG organised the cDNA bank used in this study. GHI together with KMT were responsible for designing and administering the NORCCAP clinical trial. IMBL was responsible for the pathology of the cancer cases. TI contributed with scientific input to the study. AL validated the primers and probes, carried out the RT-PCR and performed most of the statistical calculations. RL carried out the Cancer Profiling Array analysis. CM and AL drafted the manuscript. LKV helped writing the manuscript. All authors contributed to interpretation and discussion of the results and read and approved the final version.

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