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Basic Study

***NDRG2* gene copy number is not altered in colorectal carcinoma**

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Abstract

AIM

To investigate if the down-regulation of *N-myc Downstream Regulated Gene 2* (*NDRG2*) expression in colorectal carcinoma (CRC) is due to loss of the *NDRG2* allele(s).

METHODS

The following were investigated in the human colorectal cancer cell lines DLD-1, LoVo and SW-480: *NDRG2* mRNA expression levels using quantitative reverse transcription-polymerase chain reaction (qRT-PCR); interaction of the *MYC* gene-regulatory protein with the *NDRG2* promoter using chromatin immunoprecipitation; and *NDRG2* promoter methylation using bisulfite sequencing. Furthermore, we performed qPCR to analyse the copy numbers of *NDRG2* and *MYC* genes in the above three cell lines, 8 normal colorectal tissue samples and 40 CRC tissue samples.

RESULTS

As expected, *NDRG2* mRNA levels were low in the three colorectal cancer cell lines, compared to normal colon. Endogenous *MYC* protein interacted with the *NDRG2* core promoter in all three cell lines. In addition, the *NDRG2* promoter was heavily methylated in these cell lines, suggesting an epigenetic regulatory mechanism. Unaltered gene copy numbers of *NDRG2* were observed in the three cell lines. In the colorectal tissues, one normal and three CRC samples showed partial or complete loss of one *NDRG2* allele. In contrast, the *MYC* gene was amplified in one cell line and in more than 40% of the CRC cases.

CONCLUSION

Our study suggests that the reduction in *NDRG2* expression observed in CRC is due to transcriptional repression by MYC and promoter methylation, and is not due to allelic loss.

Key words: N-myc downstream-regulated gene 2; Colorectal carcinoma; MYC; Tumor suppressor; Allelic loss; Gene amplification; Copy number

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Core tip: *NDRG2* is a putative tumor suppressor gene whose expression is reduced in many cancer forms, including colorectal carcinoma (CRC). We set out therefore to investigate if down-regulation of *NDRG2* expression was due to loss of one or both alleles and/or to other mechanisms. In our paper, we show that allelic loss of *NDRG2* is a rare event in CRC. To our knowledge, this is the first study that has specifically investigated gene copy number of *NDRG2* in CRC. Furthermore, our results suggest that *MYC* is amplified in more than 40% of CRC cases. *MYC* is known to repress transcription of *NDRG2*. Our results lead us to suggest that it is the transcriptional control of *NDRG2* expression, including repression by *MYC* and epigenetic regulation, that results in decreased *NDRG2* mRNA levels in CRC, rather than allelic loss of *NDRG2*.

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INTRODUCTION

N-myc downstream regulated gene 2 (*NDRG2*) is one of four genes belonging to the *NDRG* gene family. Common for these genes is an NDR domain, a protein motif covering almost the entire protein, but the cellular functions of these genes are currently unclear^[1,2]. *NDRG2* expression has been found to be down-regulated in several human cancers including colorectal carcinoma (CRC), hepatocellular carcinoma, glioblastoma and thyroid cancer^[3-7]. *NDRG2* is a candidate tumor suppressor gene, with a better overall survival for CRC, hepatocellular carcinoma and glioma patients displaying expression of the gene compared to low or no expression^[8-12]. Further evidence of the tumor suppressor function of *NDRG2* comes from the observation that *NDRG2*-lacking mice develop various types of tumors, and from xenograft studies showing that *NDRG2*-expressing tumor cells implanted in nude mice form smaller tumors and fewer metastases than control cells^[13-15]. *NDRG2* has a number of downstream targets, including activation of phosphatase and tensin

homolog, a known tumor suppressor in the PI3K-AKT pathway^[13,16].

Several mechanisms have been suggested as possible regulators of *NDRG2* expression, of which epigenetic silencing, due to promoter hypermethylation, is the most widely observed^[4,8,9,13,14,17]. However, other regulatory mechanisms may also play a role. One example could be the transcription factor *MYC*, which is characterised as a proto-oncogene often altered in human cancers^[18]. The biological function of *MYC* seems to be to either activate or repress the transcription of target genes^[19,20]. Zhang *et al*^[21] have previously shown that ectopically expressed *MYC* is able, *via* Miz-1, to interact with and to repress transcription from the *NDRG2* promoter. Moreover, correlation of high *MYC* with reduced *NDRG2* expression has been observed in different cancers and cancer cell lines^[15,22-24]. However, an inverse relation between *MYC* levels and *NDRG2* expression seems not to apply to all cancer types^[25].

CRC is, like most other cancers, a malignant disease with a combination of both genetic and epigenetic changes. One of these changes is chromosome instability, which affects one or several chromosomal regions. Many groups have analysed changes in gene copy numbers in CRC by different approaches and found numerous chromosomal gains and losses^[26-29]. In the study by Lagerstedt *et al*^[29], the status of CRC samples classified as Dukes stages A-D was analysed, showing an increasing frequency of allelic losses at more severe stages (Dukes C and D). According to their data, allelic deletions in chromosome 14, containing the *NDRG2* gene, is already found at earlier stages (Dukes A and B) and becomes more frequent at the later stages. Although chromosome 14 is not considered one of the deletion hot spot regions, such as chromosome 8p or 18q^[27,28,30,31], we hypothesised that deletions in chromosome 14 could lead to loss of one or both of the *NDRG2* alleles. On the other hand, the *MYC* gene is found on chromosome 8q, and gains of this large chromosome arm are frequently found in CRC^[26,28,32]. Analysing the gene copy number of *MYC* is therefore of interest with regards to its possible regulatory effect on *NDRG2*.

In this study, we demonstrate a frequent increase in the gene copy number of *MYC* in CRC. In contrast, we find that changes in the copy number of the *NDRG2* locus are rare in CRC, and we suggest that reduced expression of *NDRG2* in CRC is due to epigenetic and *MYC*-related transcriptional repression.

MATERIALS AND METHODS**Cell lines and genomic DNA**

The DLD-1, LoVo and SW-480 colorectal cancer cell lines were a gift from Associate Professor Ole Vang, Roskilde University. Cells lines were incubated and maintained at 37 °C in an environment of humidified air with 5% CO₂ in McCoy's 5A + GlutaMaxTM-1 media with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Invitrogen). RNA from cell lines was purified with

the SV total RNA isolation kit (Promega) and genomic DNA was purified by ethanol precipitation after an overnight Proteinase K treatment. Reference human genomic DNA, purified from blood lymphocytes, was obtained from Roche Diagnostics, United States (Cat. No.11691112001). As a normal colonic control we used commercially available DNA (BioChain Institute Inc., D4234090). Human colon genomic DNA from tissue classified as either normal or tumorigenic was obtained from BioChain Inc, United States (Cat. no. D8235090-1; Supplementary Table S1). The commercial supplier confirms that tissue and data collection were ethically approved by their Institutional Regulatory Board and that informed consent was obtained from all human subjects.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) kit from Abcam (Ab500) was used according to the instructions, with inclusion of a final ethanol precipitation to increase the DNA concentration. Antibody against MYC (Abcam, ab56-100) was used at a concentration of 5 µg per reaction. The primers used in the PCR step were designed to cover the core promoter region in *NDRG2* (-80 to +93, Figure 1A) and their sequences were (5'-3'): CTTGAGGCATTGACCCCAGAG and CTCTTGCTGCGTCCCGAC.

Bisulfite treatment and sequencing

Bisulfite treatment of genomic DNA was performed as previously described^[33], using glycogen as carrier, and the precipitated DNA was redissolved in TE buffer, amplified by PCR and sequenced directly. The primers were designed to cover 16 CpG sites in the promoter region in *NDRG2* (Figure 1A) and their sequences were (5'-3'): TTTTCGAGGGGTATAAGGAGAGTTTATTTT and CCAAAACTCTAACTCCTAAATAACA^[34]. A positive control with *in vitro* methylated (IVM) DNA was prepared by mixing 2 µL NEB2 buffer, 1 µL 20 x S-adenosylmethionine (New England Biolabs, B9003S), 200 ng reference human genomic DNA and 1 µL SssI methyltransferase (New England BioLabs, M0226S) in a total of 20 µL. Samples were incubated at 37 °C overnight with occasional addition of 2 µL 20 x S-adenosylmethionine to ensure sufficient methyl-donor substrate. The following description was used for each CpG site: Unmethylated (no methylation signal); weakly methylated (methylation signal was less than or approximately equal to unmethylated signal); and strongly methylated (methylation signal was greater than unmethylated signal).

Quantitative real-time PCR

Determination of gene copy number was based on the LightCycler technology using SYBR Green. The sequences of the primers were (5'-3'): *NDRG2* (5' end): CCCCTTGCCTTCTAACTTCCCA and ACA-GCCCCTCCTCCACCTT; *NDRG2* (3' end): GGGG-TGAACGAAGAACAAACAAAG and CGAGGGAGAC-

GGTGAGATGAGG; *MYC*: CCAGAGGAGGAACGAGCTAA and TTGGACGGACAGGATGTATG; *GFAP*: TGACCC-TCTCCACCCCATAGTGAC and CAGCAGCAGTGCCCTGAAGATTAG; and *MECP2*: TCAGAGGGTGTG-CAGGTGAA and TTGAAAAGGCATCTTGACAAGGA. In a validation experiment using a control sample, a dilution series was produced and assayed for *NDRG2*, *MYC*, *GFAP* and *MECP2*. When C_t values were plotted against log dilution it was shown that the assays are quantitative over a range of 625-fold dilution for *NDRG2* (5' end), *NDRG2* (3' end), *MYC*, *MECP2* and 125 for *GFAP*. All samples were quantified in triplicates and mean C_t values were normalised to *GFAP* and used to calculate delta delta C_t (ddCt) relative to the reference human genomic DNA^[35]. Copy number was defined as a loss for ddCt < 0.75 and as a gain for ddCt > 1.25. Quantification of *NDRG2* mRNA expression levels in colorectal cancer cell lines, using qRT-PCR and normalisation to β -actin, was carried out as previously described^[25].

Statistical analysis

All statistical tests were carried out using GraphPad Prism 4 software and *P* values of < 0.05 were considered significant. An unpaired two-tailed *t*-test was used to compare the means of normal-distributed data for the two groups (normal vs tumor). The null hypothesis is that there is no difference between the two groups. When data of the two groups did not have equal variance, by *F* test analysis, we used a Mann-Whitney test.

RESULTS

NDRG2 expression is down-regulated in colorectal cancer cell lines

In order to examine how *NDRG2* expression is regulated in colorectal cancer, we chose to work with three cell lines. First of all, we quantified *NDRG2* mRNA levels in the three colorectal cancer cell lines DLD-1, LoVo and SW-480 and observed no or very low expression of *NDRG2*, when normalised to β -actin and compared to human colon mRNA from healthy controls (Table 1).

MYC binds to the *NDRG2* gene promoter in colorectal cancer cell lines

We were interested in seeing whether endogenous MYC was bound to the *NDRG2* promoter in these cell lines, since ectopically expressed MYC is a transcriptional repressor of *NDRG2*^[21]. A ChIP experiment did indeed show binding of endogenous MYC protein to the core promoter region of *NDRG2* in all three colorectal cancer cell lines (Figure 1B).

The *NDRG2* promoter is heavily methylated in colorectal cancer cell lines

In silico analysis of the *NDRG2* promoter predicted a CpG island between -380 and +1471 relative to the transcriptional start site (%GC = 66.3, observed/expected CpG = 0.673, cpgislands.usc.edu/cpg.aspx).

Figure 1 Epigenetic and chromatin immunoprecipitation analysis of the *NDRG2* promoter in three colorectal cancer cell lines. A: The *NDRG2* gene sequence around the transcriptional start site at +1. Primer-binding regions for PCR are underlined and CpG sites subjected to methylation analysis are numbered 1 to 16; B: Endogenous MYC interacts with the *NDRG2* core promoter. ChIP analysis was carried out on SW-480, LoVo and DLD-1 cell extracts using antibody against the transcription factor MYC. "No antibody" was without antibody and "input" served as a positive control. Genomic DNA was used as positive control for the PCR reaction; C: The *NDRG2* promoter is hypermethylated in three colorectal cancer cell lines. Bisulfite sequencing was carried out on human genomic DNA from LoVo, DLD-1 and SW-480 cell lines, normal colonic DNA, reference DNA and *in vitro* SssI-methylated (IVM) DNA. Each CpG site was rated as unmethylated, weakly methylated ($\leq 50\%$ methylated), or strongly methylated ($> 50\%$ methylated).

Table 1 Mean values of normalised levels of *NDRG2* mRNA in colorectal cancer cell lines and healthy colonic tissue

Sample	mRNA level
DLD-1 cell line	0
LoVo cell line	0.005
SW-480 cell line	0.001
Control human colon ^a	0.034 ± 0.009

All samples were analysed in technical triplicates and normalised to β -actin mRNA levels. ^aPreviously published data for the mean \pm standard deviation for 15 individuals^[3].

To establish the methylation status of the *NDRG2* proximal promoter in all three cell lines, we carried out bisulfite treatment and sequencing of the region from -426 to -107, which contains 16 CpG sequences. Bisulfite treatment converts all unmethylated cytosines into uracils, while cytosines with a methyl group attached remain unaltered. As controls, we compared our results with healthy colon genomic DNA, reference genomic DNA from normal blood lymphocytes, and IVM genomic DNA. As presented in Figure 1C, the normal colon genomic DNA and reference genomic DNA sample were predominantly weakly methylated, whereas the *in vitro* methylated control was completely methylated at all cytosines. The three colorectal cancer cell lines, LoVo, DLD-1 and SW-480, displayed strong methylation at the majority of CpG sites (Figure 1C).

***NDRG2* gene copy number is not altered in colorectal cancer**

We wished to determine the allelic copy numbers of both *NDRG2* and *MYC* in human colorectal carcinoma. By combining qPCR with the mathematical delta delta C_t equation (ddCt), we were able to quantify both losses and gains of these genes. Our experimental setup was validated by analysing the copy numbers of the X-chromosome linked *MECP2* gene in males and females - with the expected one and two X-chromosomes, respectively. As visualised in Figure 2, DNA from 3 females were scored with a ddCt value close to 1.00, which means that the same gene copy ratio between *MeCP2* and *GFAP* was present in both the analysed samples and the reference female genomic sample. A ddCt value of 1.00 therefore represents the normal two alleles. On the contrary, males displayed a ddCt value of approximately 0.50, which represents one allele. Finally, we tested our setup on an unknown sample clearly showing the pattern for male DNA. The conclusion was, therefore, that our setup clearly could differentiate between females and male, *i.e.*, one and two alleles, and has the potential to analyse the copy numbers of *NDRG2* and *MYC*.

We have previously published data showing a statistically significant down-regulation of *NDRG2* mRNA in CRC^[3], and the main aim in the present study has therefore been to analyse if allelic loss of *NDRG2* could explain cases of decreased *NDRG2* mRNA levels. For a thorough investigation of *NDRG2*, we selected two

regions of the genomic sequence of *NDRG2*, one lying in the 5' part of the sequence and the other lying in the 3' end. We first analysed the three colorectal cancer cell lines for both *NDRG2* and *MYC* and found no changes in the copy number of *NDRG2*, in contrast to *MYC*, for which we observed copy number loss in the LoVo cell line, the normal two alleles in DLD-1 cells and a clear copy number gain in SW-480 (Table 2). This latter result is in agreement with a previous study showing a 5 to 10-fold genomic amplification of *MYC* in SW-480 cells^[36].

We next analysed 8 normal and 40 CRC tissue samples. In one case out of the eight normal samples, our data indicated copy number loss at the 5' end of the *NDRG2* gene; otherwise, none of the samples showed any copy number alterations for *NDRG2* (Table 3). As summarised in Table 3, 29 out of the 40 CRC samples (72%) had an unaltered copy number, 2 samples showed loss at either the 5' or the 3' end of *NDRG2*, and only in one case did we observe loss at both ends of the gene. In contrast, we found complete copy number gain of *NDRG2* in 3 cases and partial gain in 9 cases (Supplementary Table S2).

Finally, we determined the copy numbers of *MYC* in the same 8 normal and 40 CRC samples, and observed one case of genomic amplification in the normal samples. Otherwise, we did not find any allelic changes in the normal samples (Table 3). For the 40 CRC samples, we observed copy number loss in 4 cases, the normal two copies in nearly half the cases (19 out of 40), and copy number gains of the *MYC* gene in the remaining 17 samples (42.5%) (Supplementary Table S1). However, the observed differences in copy number between normal and CRC tissue did not reach statistical significance (Mann-Whitney test, Table 3).

DISCUSSION

We and others have previously published data showing a statistically significant reduction in *NDRG2* mRNA levels in CRC compared to normal colorectal tissue samples^[3,12,23]. Similar findings have been observed in other cancers including gliomas, hepatocellular carcinoma, breast cancer, thyroid cancer and meningioma^[5-7,25,37]. Exactly how and why *NDRG2* expression is reduced is not fully understood, but repression by the *MYC* transcription factor is likely to be involved in some cases, just as promoter hypermethylation seems to play an important role^[4,14,21,34]. Here, we show that 16 potential methylation sites in the proximal promoter of *NDRG2* are heavily methylated in all three colorectal cancer cell lines tested. Methylation of the analysed region from -426 to -107 could reduce accessibility to the transcription factors WT1 and HIF1 α , which have binding sites in this region^[38,39] and/or result in transcriptional silencing. In support of this, previous studies have shown that reversal of methylation by 5-aza-2'-deoxycytidine treatment leads to increased *NDRG2* mRNA levels in the colorectal cancer cell lines CaCo2, HCT116 and SW480^[34]. Furthermore,

Table 2 ddCt values and corresponding copy numbers for the *NDRG2* and *MYC* genes in colorectal cancer cell lines

Cell line	<i>NDRG2</i> - 5' end		<i>NDRG2</i> - 3' end		<i>MYC</i>	
	ddCt \pm SD	Copy number	ddCt \pm SD	Copy number	ddCt \pm SD	Copy number
LoVo	1.23 \pm 0.47	2	1.12 \pm 0.51	2	0.91 \pm 0.31	2
DLD-1	1.04 \pm 0.23	2	1.08 \pm 0.50	2	0.74 \pm 0.22	Loss
SW-480	1.04 \pm 0.26	2	0.94 \pm 0.44	2	4.88 \pm 0.30	Gain

Copy number loss is defined as ddCt < 0.75 and a gain is defined as ddCt > 1.25. ddCt: Delta delta Ct; SD: Standard deviation.

Table 3 Alteration in copy numbers for the *NDRG2* and *MYC* genes in colorectal tissue

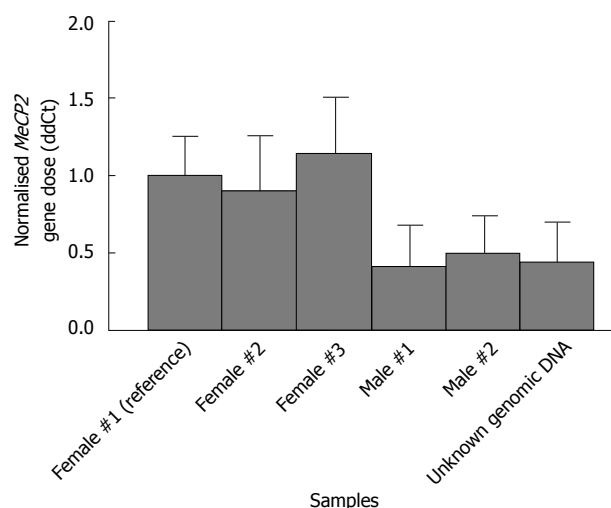
Colorectal tissue	Number of samples	Loss ddCt < 0.75	Unaltered ddCt 0.75-1.25	Gain ddCt > 1.25	Normal vs CRC
NDRG2 - 5' end					
Normal	8	1	7	0	P = 0.194 ^a
CRC	40	2	29	9	
NDRG2 - 3' end					
Normal	8	0	8	0	P = 0.470 ^a
CRC	40	2	32	6	
MYC					
Normal	8	0	7	1	P = 0.135 ^b
CRC	40	4	19	17	

^a P value for comparison of ddCt values (supplementary table S2) in normal and CRC samples using an unpaired two-tailed t test; ^b P value for comparison of ddCt values (supplementary table S2) in normal and CRC samples using a Mann-Whitney test. ddCt: Delta delta Ct.

DNA methylation at the *NDRG2* promoter was shown to be significantly higher in CRC tissue compared to normal colonic tissue from the same patients^[14,34].

Our ChIP experiments on three colorectal cancer cell lines showed that endogenous *MYC* interacts with the *NDRG2* core promoter. Although *MYC* is considered a classical transcription factor, it is also involved in the maintenance of chromatin structure^[40,41]. For example, *MYC* has been shown to recruit DNA methyltransferase 3a to the promoter region of a gene to exert its repressive activity^[42]. Thus, we suggest that *MYC* could be involved in the regulation of *NDRG2* by recruitment of other proteins to produce an epigenetic silencing of *NDRG2*.

However, the suggested regulatory mechanisms cannot explain all cases of down-regulation of *NDRG2* expression, and we were therefore interested in looking at allelic loss to see if this genetic event could contribute to the decreased *NDRG2* mRNA levels observed in CRC. To investigate this question, we designed an experimental setup making it possible to quantify the copy numbers of any gene. In a validation experiment, we could easily differentiate between one or two copies of the X-chromosome linked gene *MECP2*. Our data indicate that allelic loss at the *NDRG2* locus is not very frequent in CRC. On the contrary, a subset of CRC cases showed gains of one or both ends of the *NDRG2* gene, which might lead to elevated levels of *NDRG2* mRNA. These findings were unexpected, since allelic losses in chromosome 14 are more frequently observed than gains^[27,28]. Although we have only looked at copy number

**Figure 2** Validation of the gene copy number experimental setup. Bar diagram showing the calculated delta delta Ct values (ddCt) of the X-linked *MeCP2* gene normalised to *GFAP*, giving the expected result (one copy in males and two copies in females). A ddCt value of 1.00 in the reference female genomic sample represents the normal two alleles. Data are presented as mean (filled bars) and SD (whiskers).

changes in CRC, our results might be applied to other cancers and could explain why we observed an increase in *NDRG2* levels in approximately 8% of 154 paired normal and tumor samples analysed from 19 different tumor types^[25].

The proto-oncogene *MYC* is located on chromosome 8 at the q24.12 region, and several groups have shown amplification of chromosome 8q^[27,28,43]. Indeed, we observed an increase in *MYC* gene copy numbers in nearly every second CRC sample, confirming a frequent gain at this particular gene locus. However, we did not detect the same high percentage of *MYC* amplification as a previous study focusing on the 8q24 region, which revealed that nearly 80% of the cases analysed had some kind of gene amplification^[32]. Since *MYC* has the potential to repress *NDRG2* transcription^[21], increased copy numbers of the *MYC* gene could lead to higher levels of *MYC* protein and thereby a reduced level of *NDRG2* mRNA.

Finally, copy number loss of the 5' end of *NDRG2* and a gain of *MYC* were observed in separate normal samples and might indicate a rare, but real, genomic alteration in healthy tissue. An alternative explanation is that since all normal samples were obtained from patients diagnosed with CRC and classified as normal, the tissue might be at an early pre-malignant stage with

no visual changes, but where genetic abnormalities had already occurred.

In conclusion, we observed *NDRG2* promoter hypermethylation and interaction of endogenous MYC with the core promoter in three colorectal cancer cell lines, together with absent or low *NDRG2* mRNA expression. Frequent allelic loss was not found at the *NDRG2* locus in the colorectal cancer cell lines and tissue samples from either normal or tumor tissues. In contrast, we observed partial or complete *NDRG2* copy number gains in more than 25% of the CRC cases, compared to none in the normal samples. We also found that more than 40% of CRC cases displayed *MYC* amplification, which indicates that the level of *MYC* mRNA is elevated in CRC. We conclude that epigenetic silencing and transcriptional repression by MYC are likely to be more important than copy number loss for the reduced levels of *NDRG2* mRNA observed in CRC.

COMMENTS

Background

A frequent change observed in colorectal carcinoma (CRC) is chromosomal instability, in which gain or loss of chromosomal regions affects levels of gene expression. Thus, loss of one or both alleles could explain the reduced expression of tumor suppressor genes, such as *NDRG2*, that is observed in CRC. Alternatively, *NDRG2* down-regulation could be due to transcriptional and epigenetic mechanisms.

Research frontiers

In order to understand the origin of CRC, it is important to investigate changes at the epigenetic, genetic and transcriptional level. This study investigated regulation of *NDRG2* gene expression using bisulfite-sequencing to study gene methylation, quantitative polymerase chain reaction to study gene copy number as well as chromatin immunoprecipitation to study DNA-binding of the endogenous gene-regulatory protein MYC.

Innovations and breakthroughs

This study shows for the first time that gene copy number for *NDRG2* is unaltered in CRC cell lines and clinical samples.

Applications

The authors describe a validated approach to determine gene copy number, relative to a control gene, using the comparative (ddCt) approach. Future approaches could focus on re-activating expression of *NDRG2* in CRC.

Terminology

NDRG2 is a newly described tumor suppressor gene that is down-regulated in a large range of cancers, including CRC. Interest in *NDRG2* as a therapeutic target is supported by studies showing a better prognosis in patients having higher *NDRG2* expression in tumor tissues.

Peer-review

The paper is very good.

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