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Review

NDRG2: A Candidate Tumor Suppressor Gene in Search of a Function

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Abstract

NDRG2 is a recently described member of the *NDRG* family, with suggested roles in neurodegenerative disease, cancer and cell differentiation. Expression levels of *NDRG2* have been shown to be reduced or absent in a range of cancer cell lines and tumor types, leading to a suggested function as a tumor suppressor gene. This is supported by two key observations: re-expression of NDRG2 protein in cancer cell lines leads to a reduction in cell growth, and expression levels of *NDRG2* mRNA correlate inversely with the clinical grade of various tumors. However, very little is currently known about the function of the NDRG2 protein. All members of the *NDRG* family share an α/β-hydrolase fold, but the amino acids of the catalytic triad are poorly conserved, suggesting that the NDRG protein members lack enzymatic activity. In this review, we will summarise the current knowledge on *NDRG2* with respect to cancer, including expression patterns and regulation, possible links to apoptotic signalling pathways, hypoxia, cell cycle control and finally, how we can use this information on *NDRG2* with regards to cancer management.

Keywords: NDRG2, MYC, cancer, tumor suppressor, miRNA, apoptosis, cell cycle
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1. INTRODUCTION

According to the World Health Organization (WHO), approximately 13% of all deaths worldwide are caused by cancers (http://www.who.int). Although we have made substantial progress in the treatment of cancer patients, our knowledge and understanding of the biology of human cancers is still limited. Particularly during the last few decades, our knowledge of the genes and pathways involved in carcinogenesis and the role of genetic events, epigenetic modifications and the action of non-coding elements such as microRNAs in the transformation of normal cells into cancer cells, has expanded greatly. Two groups of cancer-related genes are tumor suppressor genes and oncogenes, including but not limited to well-known genes such as p53, APC, BRCA1 and Myc. Furthermore, we are still discovering links between new genes or gene families and human cancer.

One example of such a gene family is the *N-myc Downstream Regulated Gene* (*NDRG*) family composed of four members designated *NDRG1* to *NDRG4*. The first member, *NDRG1*, was identified in 1996 by Kokame and co-workers and in 2001 a search in a human EST database revealed *NDRG2*, *NDRG3*, and *NDRG4* as genes with sequence homologies between 57–62% to that of *NDRG1*[1–3]. The *NDRG* genes are located on four different chromosomes, and they encode proteins of varying sizes ranging from open reading frames of 339 to 394 amino acids, and with *NDRG1* possessing a unique 14 amino acid sequence and *NDRG3* the remnants of a glycosyl transferase family 2-type domain (Figure 1). Common for all four genes is an α/β-hydrolase fold domain with a parallel stretch of eight β-strands, but where two out of the three residues in the catalytic triad are non-conserved[4]. So

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far, it has not been possible to elucidate if this protein domain actually has a biological function such as those found in other α/β-hydrolase domain-possessing proteins (*e.g.* lipases). However, the 3D-structures of the NDRG proteins from both mouse and human are highly similar, indicating conserved and thereby important functions (Crystal structures of mouse and human NDRG2 are located at `http://www.rcsb.org/pdb/explore/explore.do?structureId=2QMQ` and 2XMR[5]).

Aside from NDRG1, none of the family members have been directly connected to any biological or physiological function. In the case of *NDRG1*, two disease-causing mutations have been identified which result in an altered NDRG1 protein, either due to exon skipping or a premature-termination codon. Both kinds of mutations have been directly linked to a severe form of Charcot-Marie-Tooth Disease, a demyelinating disorder[6, 7]. NDRG2 protein is closely associated with the plaques and tangles that characterise Alzheimer's Disease, but its role in neurodegeneration is unclear[8]. Finally, all family members have, to a varying degree, been connected with human cancer although it is not known if this occurs as a consequence of cancer or through a direct involvement in the etiology of the cancers. It should be emphasised that there is currently no evidence of DNA sequence alterations of any kind in the *NDRG* genes in human cancer. In the following we will review the current knowledge about *NDRG2* and human cancers.

2. EXPRESSION OF NDRG2 IN HUMAN CANCER

Since the beginning of 2003, numerous papers have been published showing the involvement of *NDRG2* in cancer. One of the first indications was presented by Deng *et al.*, showing the ability of NDRG2 to inhibit the proliferation of glioblastoma cells[9]. They were also the first to show a difference in *NDRG2* expression related to tumor grade classification. These observations in glioblastoma were later verified[10]. Additionally, down-regulation of *NDRG2* mRNA in tumor tissues, compared to normal tissues, have also been found in other cancers including liver and pancreatic cancer[11, 12], meningioma[13], colorectal cancer[14, 15], thyroid cancer[16, 17], clear cell renal cell carcinoma[18], breast cancer[16] and finally, low levels of *NDRG2* expression were observed in oral squamous-cell carcinoma[19] and in esophageal squamous cell carcinoma[20]. Several microarray studies have added information about the expression of *NDRG2* in human cancers and based on these array data, *NDRG2* expression was found to be down-regulated in breast cancer, colorectal cancer and squamous cell carcinoma samples compared to normal tissue samples[21–23]. Finally, a study on extraskeletal myoxid chondrosarcomas (EMCs) has shown that the level of *NDRG2* mRNA is up-regulated in EMCs compared to other sarcomas[24]. The study by Filion *et al.* is one of the first studies showing up-regulation of *NDRG2* in human cancers. However, this might not be an uncommon phenomenon, since an increase of 2-fold or more in mRNA levels was observed in approximately 8% of tumors compared to their normal counterparts, when analysing 154 paired normal-tumor samples from 19 different cancers[16].

2.1. Is NDRG2 a new tumor suppressor gene?

Cancer and cancer-related genes are normally divided into two groups depending on whether they protect against or promote carcinogenesis. The more protective genes have historically been named tumor suppressor genes (TSG) and the genes promoting carcinogenesis are called oncogenes or proto-oncogenes. There exist numerous definitions for what constitutes a TSG, but common for these is that inactivation or partial loss of a TSG will increase the risk of developing cancer. Although classification of a gene as a TSG is a matter of interpretation, *NDRG2* should be considered a new candidate TSG at the current time, mainly due to its ability to significantly reduce the growth of tumor cells compared to cell lacking *NDRG2* expression as found in many cases and from different cancers[9, 19, 20, 25–27]. The second argument for defining *NDRG2* as a TSG, explained below, is the observation that cancer patients exhibiting expression of the gene in tumor tissue have a much better overall survival compared to patients lacking or experiencing reduced *NDRG2* levels.

In 2008, Lee *et al.* were the first to present data arguing for *NDRG2* as a gene with the potential to reduce the mobility and colonisation of cancer cells, and in a mouse model system they further showed that cancer cells expressing *NDRG2* gave significantly reduced distant metastatic formation in the liver compared to control cells lacking *NDRG2*[12]. Other groups have later verified the potential of *NDRG2* to reduce the adhesion, invasion and migration of cancer cells as seen in breast cancer cells[28, 29], fibrosarcoma cells[30] and in renal cell carcinoma[31]. These observations could implicate *NDRG2* as a candidate metastatic suppressor gene (MSG), and these observations are supported by reports showing involvement of *NDRG2* in the negative regulation of various matrix metalloproteases (MMPs) known to degrade the extracellular matrix and

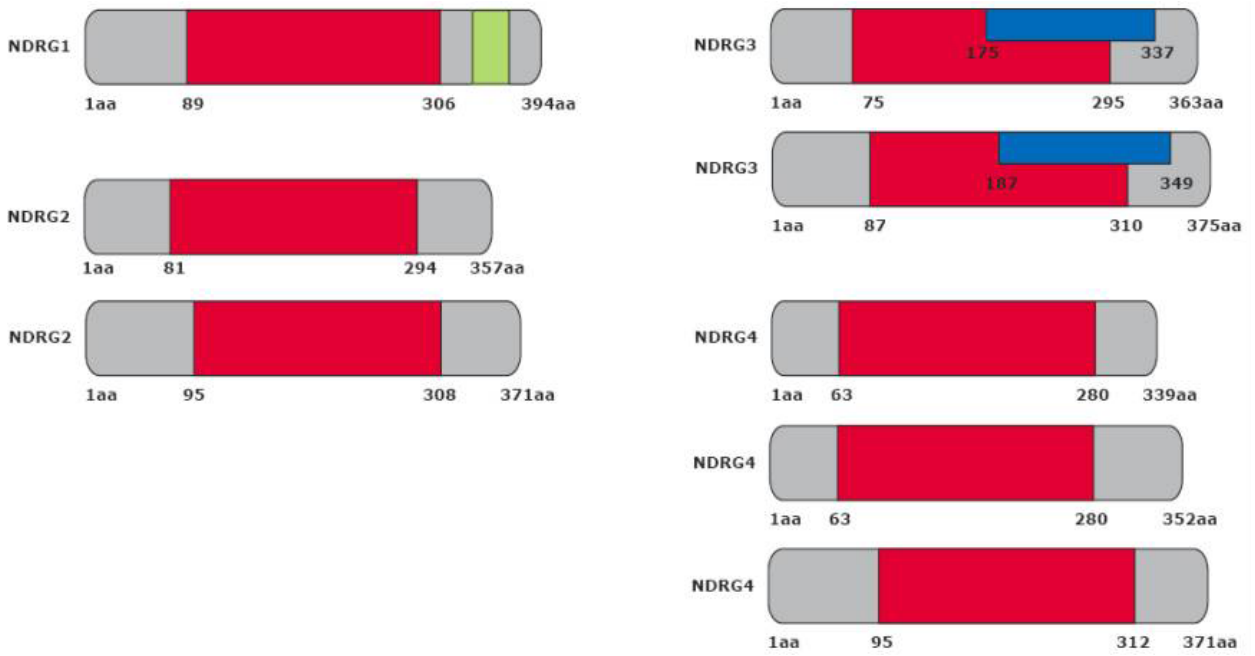

Figure 1. NDRG family members and their isoforms due to alternative splicing. Classification and orientation of the various domains are based on sequence analyses in the CLC workbench (http://www.clcbio.com) with standard conditions. Red boxes indicate alpha/beta-hydrolase domains, the green box indicates a three tandem repeat unique for NDRG1 and blue boxes represent the remnants of glycosyl transferase family 2 type domain.

therefore to be important when cancer cells undergo metastasis[12, 28]. The classic difference between a TSG and a MSG is that a MSG has no direct effect on the primary tumor, but can reduce or inhibit metastasis of primary tumor cells. So to argue that *NDRG2* is a classic MSG is not straightforward. However, it is possible that there exists a small group of genes, which possess both tumor and metastatic suppressive activities, of which *NDRG2* is one, but it is more likely that the tumor suppressive actions of *NDRG2* are responsible for the observed metastatic properties.

2.2. *Regulation of NDRG2 expression in human cancers*

The majority of tissue types examined with respect to *NDRG2* mRNA and protein show a reduced level in tumor samples compared to normal tissue samples, and it is therefore interesting to learn more about how the expression of *NDRG2* is regulated. At least three different mechanisms of *NDRG2* inactivation are known today: transcriptional repression by the MYC transcription factor, epigenetic silencing by promoter methylation and post-translational inactivation by at least one microRNA (Figure 2).

In 2006, Zhang *et al.* demonstrated that MYC can interact with the *NDRG2* promoter and furthermore repress *NDRG2* expression *via* Miz-1[32]. Furthermore, they found an inverse correlation between NDRG2 and MYC protein levels in various cancer cell lines. These findings have inspired others to investigate if this repressive mechanism also applies to tissue samples. Inverse correlations between *NDRG2* and *Myc* expression have been observed in colorectal carcinoma[33], thyroid cancer[17] and in esophageal squamous cell carcinoma[20]. However, in some cases it has not been possible to verify the previously observed correlation as in thyroid cancer[16]. An inverse correlation was also lacking for breast cancer[16]. Discrepancy in the observed results may be due to different sample sizes, tissue-specific mechanisms for NDRG2 inactivation and/or different ethnic origin of the samples. However, it is worth emphasising that even in tissues where an inverse correlation of *NDRG2* and *Myc* expression has been demonstrated, this is not proof that elevated levels of *Myc* are responsible for the decreased *NDRG2* expression in cancers.

The *NDRG2* promoter possesses a CpG island, as do the rest of the *NDRG* family members, and may be sub-

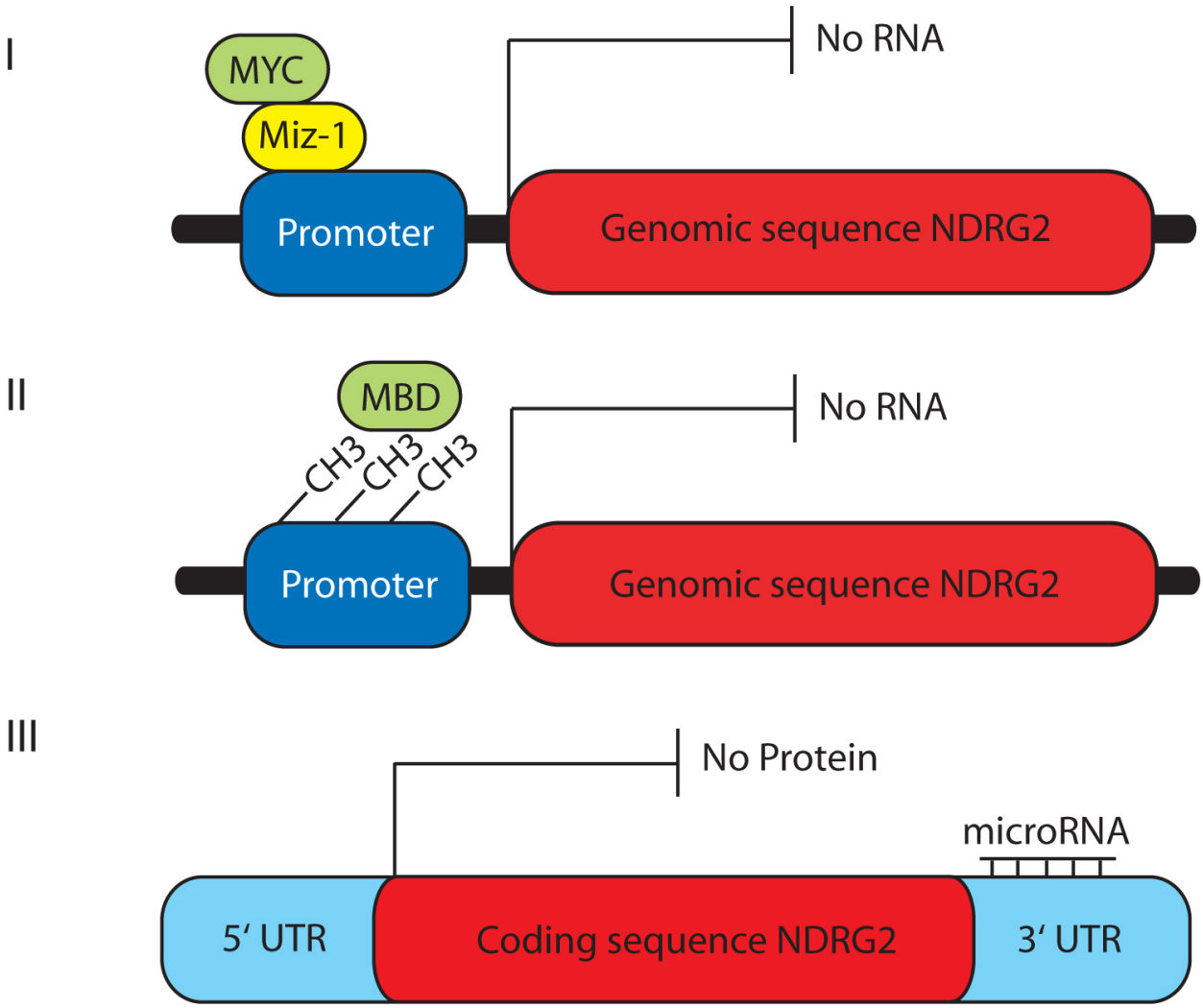

Figure 2. Mechanisms of NDRG2 inactivation. Schematic presentation of the proposed mechanisms involved in NDRG2 inactivation: (I) inactivation at the transcriptional level by MYC in association with Miz-1, (II) epigenetic silencing by promoter hypermethylation and (III) inactivation by the action of a microRNA on *NDRG2* mRNA. MBD, Methyl-CpG binding protein.

jected to epigenetic silencing through DNA methylation. Using various techniques like methylation-specific PCR and bisulfite sequencing, it has been possible to determine the degree of *NDRG2* promoter methylation, and in a number of studies using different cancer cell lines and tissues such as liver cancer, primary glioblastoma, aggressive meningioma, oral squamous-cell carcinoma and colorectal cancer, promoter methylation has been observed[10, 12, 13, 15, 19]. Those studies also showed that the higher the degree of promoter methylation, the lower the level of *NDRG2* expression and additionally, that more aggressive or higher grade tumors had a higher degree of methylation at the *NDRG2* promoter and thereby the lowest level of *NDRG2* mRNA. A way to re-activate an epigenetically silenced gene is to use the demethylating agent 5'-aza-2'-deoxycytidine (5'aza-dC), often in combination with the histone deacetylation agent Trichostatin A (TSA). Results from different cancer cell lines have shown that the expression of *NDRG2* can be re-activated by 5'aza-dC alone, but is more pronounced when combined with TSA[15, 19, 34].

One of the exciting advances in cancer research is the discovery of microRNAs whose expression is altered in human cancer. *In silico* analysis predicts that *NDRG2* is a target gene for microRNA regulation, with over 100

potential binding sites for microRNAs (http://www.microrna.org). *NDRG2* has recently been found to be a direct target gene of microRNA-650, which acts *via* a sequence in the 3' untranslated region of the *NDRG2* mRNA[34].

It is possible that the expression of NDRG2 in human cancer is regulated by other mechanisms than those currently known and described above. Additionally, NDRG2 is subjected to post-translational modifications, such as phosphorylation, as is seen for the other NDRG proteins[35–37]. Although the mechanisms controlling post-translational modification of NDRG2 are still unclear, a study by Kim *et al.* showed that mutation of three phosphorylation sites abolished the ability of NDRG2 to reduce growth of cancer cells that is normally seen for the wild-type protein[26]. Further research is necessary to investigate if the function or activity of NDRG2 is altered in cancer, *e.g.* due to currently unknown mutations in the NDRG2 coding sequence, and to determine if *NDRG2* gene copy losses exist, which would lead to the decreased mRNA levels observed in many human cancers.

3. FUNCTION OF NDRG2 IN CANCER

3.1. A role for NDRG2 in apoptosis?

In multicellular organisms such as humans, apoptosis or programmed cell death is an important and precisely regulated process that occurs throughout life. When talking about cell death, it is common to describe apoptosis on one hand as active programmed cell death, and necrosis on the other hand as a passive process. There exist two main apoptotic pathways, an extrinsic and an intrinsic pathway. Activation of the extrinsic pathway occurs by binding of external signals, *e.g.* ligands, to cell surface receptors of which the tumor necrosis factor (TNF) receptor family, also known as death receptors, is one of the best known receptor type, binding ligands like Fas and TNF-α[38]. The other apoptotic pathway is the intrinsic pathway where oxidative stress and exposure to chemicals are examples of stimuli activating this pathway. The intrinsic apoptotic pathway often includes mitochondria and is also called the mitochondrial pathway[39]. Apoptosis is basically a way for an organism to remove cells that are no longer important as seen during development, but it is also a way to remove damaged or altered cells. Since tumor cells often possess one or several genetic changes making them abnormal, they are targets for removal by apoptosis. However, many tumor cells have gained the capacity to avoid the apoptotic pathways by anti-apoptotic strategies.

p53 plays an important role in the intrinsic apoptotic pathway where it stimulates cell cycle arrest upon detection of DNA damage; in cases where DNA damage cannot be repaired, p53-mediated apoptosis is observed[40]. Furthermore, p53 is inactivated in many human cancers due to mutations, deletions or other mechanisms, making inactivation of p53 one of the key events in the anti-apoptotic tumor strategy[41]. NDRG2 has been linked to p53-mediated apoptosis, first of all by being a direct target gene of the transcription factor p53. In a study by Liu and colleagues, they presented evidence that p53 was able to bind to and activate the *NDRG2* gene promoter, and they also showed that increasing levels of p53, *e.g.* due to stress, led to induction of *NDRG2* expression[42]. Using fluorescence-activated cell sorting (FACS) to measure the number of apoptotic cells, several studies have shown that forced over-expression of *NDRG2* resulted in a larger percentage of apoptotic cells compared to control cells lacking *NDRG2*[27, 42–44]. Taken together, *NDRG2* seems to play an active role in apoptosis, but the specific function of the gene still has to be elucidated.

3.2. A role for NDRG2 in regulating the cell cycle and cell proliferation?

A central position in carcinogenesis must be the cell cycle, since defective control of the cell cycle is seen in every cancer. As put forth by Hanahan and Weinberg, four of the six hallmarks of cancers have to do with growth: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replication potential and evasion of apoptosis[45].

Cells placed in the G_0 phase of the cell cycle can enter the cell cycle and the G_1 phase by mitogenic stimulation. Once cells have entered the G_1 phase they will proceed until they reach the restriction point at the end of G_1, and if they pass beyond this point they are destined to continue throughout the S-, G_2 and the M-phase of the cell cycle. The components driving the cell cycle forward are mainly cyclins and cyclin-dependent kinases (CDKs), which can form different complexes depending on which phase of the cell cycle a cell is in. Transfection of colorectal and breast cancer cell lines with a NDRG2 expression vector indicated that over-expression of NDRG2 resulted in the induction of p21 and decreased levels of Cyclin D1[26, 46]. Both proteins are important players in regulating the cell cycle by controlling the progression from G_1 phase into the S-phase, in the case of Cyclin D1, whereas p21 seems to inhibit the progression from G_1 to S-phase[47, 48]. A proposed model for this regulation by NDRG2 is shown in Figure 3.

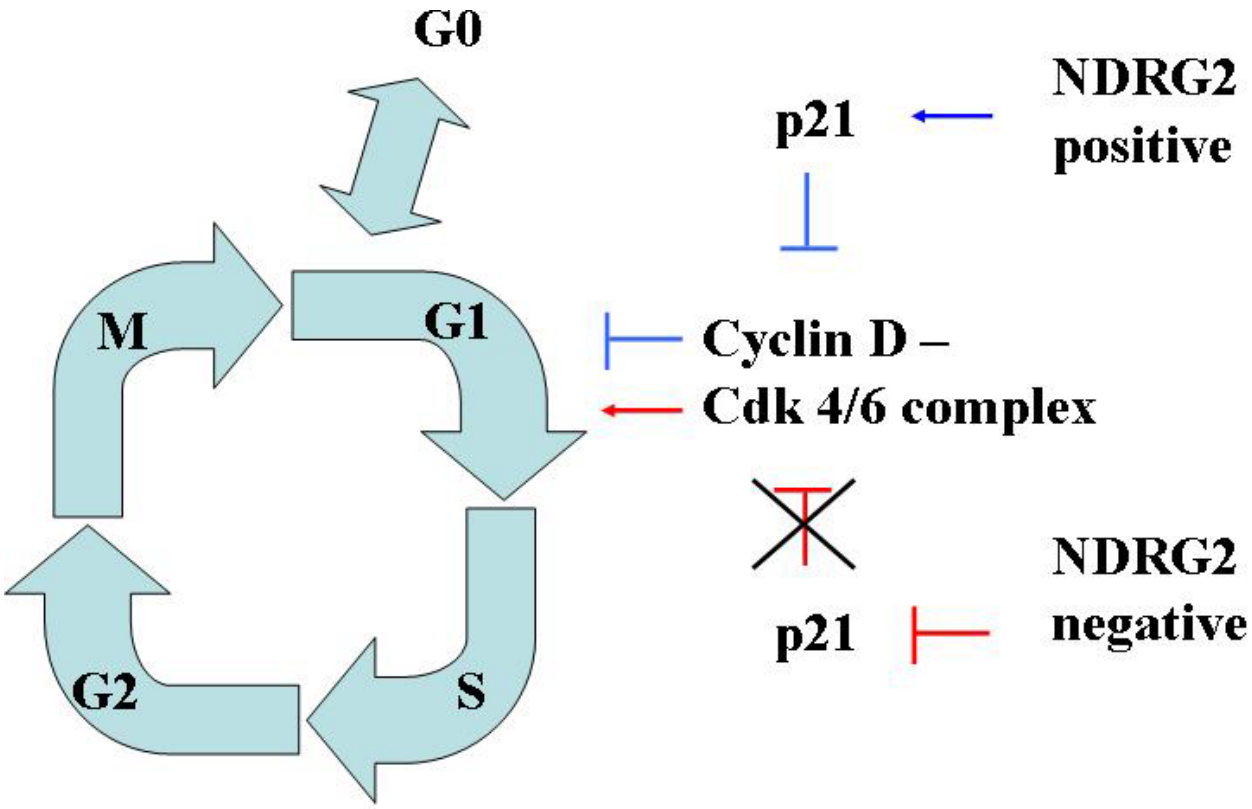

Figure 3. Proposed model for NDRG2 action on the eukaryotic cell cycle. The presence or absence of NDRG2 either inhibits or maintains the function of G_1 phase-specific cyclin D–Cdk 4/6 complex *via* p21. If the cyclin D–Cdk 4/6 complex is inhibited, *via* NDRG2-induced up-regulation of p21, cells will exhibit a reduced growth (blue lines), whereas low levels of p21 will drive the cells into the S-phase of the cell cycle by the action of the cyclin D–CdK 4/6 complex (red lines).

The significance of *NDRG2* expression for cell proliferation has been examined in various cancer cell lines. From proliferation studies in breast cancer cells, it was observed that over-expression of *NDRG2* reduced the number of MDA-MB-231 cells compared to mock transfected cells[46]. Similar findings were observed in oral squamous-cell carcinoma cells, colorectal carcinoma cells, gastric cancer cells, melanoma cells and glioblastoma cells[9, 19, 25, 26, 30]. Other studies examining over-expression of *NDRG2* revealed less aggressive cells with a slower migration rate, but also cells that metastasised at a lower frequency than cells lacking NDRG2[12, 28]. Finally, the overall survival for patients suffering from liver cancer, gastric cancer and esophageal squamous cell carcinoma was much improved for tumors which retained high *NDRG2* expression than those without *NDRG2*[12, 20, 25]. Additionally, as mentioned earlier, mutations of three phosphorylation sites in the protein sequence of NDRG2 abolished its ability to reduce the growth of cancer cells. Taken together, the above mentioned observations point towards an important role of NDRG2 in regulating cell proliferation and cellular behaviour.

3.3. NDRG2 and hypoxia

Solid tumors are frequently exposed to low levels of oxygen, a phenomenon termed hypoxia. Under a hypoxic state, the expression profile of various genes becomes altered and different cellular processes are altered. A key player during hypoxia is the transcription factor Hypoxia-inducible factor 1 (HIF-1), which is a mediator of hypoxia-mediated signalling. *NDRG1* was previously found to be altered during hypoxia and is, in some cases, regulated by HIF-1[49]. In 2008, Wang *et al.* were the first to show that *NDRG2*, as well, was altered during hypoxic signalling[50]. They found that *NDRG2* mRNA and protein increased under hypoxia and hypoxia-like conditions, and that HIF-1 is able to bind to Hypoxia responsive elements (HREs) found in the *NDRG2* promoter. Recently, the same

group presented further evidence that NDRG2 and HIF-1 contribute to hypoxia-mediated responses. In the work by Liu *et al.*, they verified first that *NDRG2* was up-regulated during hypoxia, but also found *NDRG2* to be up-regulated by radiation[51]. Furthermore, it was observed that NDRG2 influenced the hypoxia-induced radioresistance and radiation-mediated apoptosis. Whereas it seems clear that *NDRG2* is changed under hypoxic conditions, the physiological significance is not completely understood. Further analyses are required, just as we need to understand how NDRG2, on the one hand, can be beneficial when looking at the overall survival rate of cancer patients, and at the same time have a protective role against *e.g.* radiation-induced apoptosis.

4. PERSPECTIVES FOR CANCER TREATMENT

Even though we still lack a concrete function for NDRG2, data continues to accumulate that argue strongly for *NDRG2* as a candidate tumor suppressor gene with an important function in human cancer. The current research into *NDRG2* is roughly concentrated in two branches. First of all, several groups are measuring the levels of *NDRG2* expression in normal human and tumor tissue samples and further trying to understanding the mechanisms underlying the often observed down-regulation of its mRNA. Alternatively, recent research has focused on *NDRG2* at a more functional level with the clear goal to place *NDRG2* in a specific signal pathway or within a specific biological process such as the cell cycle.

The information that is obtained by looking at expression data from both normal and tumor tissues can be very valuable in the development of new and better diagnostic tools, as well as in the management and treatment of human cancers. From all the cancers analysed, it is striking how often the expression of *NDRG2* is down-regulated, and in several reports the altered *NDRG2* expression has been compared to different clinicopathological features and tumor classifications. When summarising the available data, the conclusion is that the worse a tumor is classified or the later stage the tumor is in, the lower the expression of *NDRG2*[9, 10, 13, 14, 52, 53], but also that *NDRG2* expression correlates with features such as differentiation[54]. These observations open up for *NDRG2* as a potential new marker in tumor diagnostics, although further research is warranted. At a more prognostic level, there seems to be a connection between the level of *NDRG2* in tumor tissue and the survival of cancer patients. However, the survival length depends on the cancer type[10, 20, 25, 53]. In two recent papers, it has been suggested that *NDRG2* could serve as a prognostic marker in human astrocytomas and for the prediction of colorectal cancer relapse[54, 55]. Before using *NDRG2* as a prognostic marker, however, additional studies are needed, primarily analysing more cancer patients with regards to relapse, metastasis, tumor stages *etc.*

On the more therapeutic side of cancer management, it is worth considering whether re-activation of *NDRG2* expression would be possible and beneficial for tumors lacking *NDRG2* expression. Since the gene is inactivated by promoter hypermethylation in many cases, and methylation is a reversible process, re-activation may be possible at least in theory. Another possible way to overcome a reduction of *NDRG2* expression is to understand the specific pathway(s) that the gene is involved in, and to develop therapeutics targeting genes or gene products up- or downstream of *NDRG2*. In this fashion we might be able to restore the implicated signal pathway and circumvent the lack of *NDRG2*.

5. CONCLUSIONS

The more we learn about the expression and possible functions of *NDRG2*, the more apparent it becomes that we are dealing with a new candidate tumor suppressor gene. Although NDRG2 has an unknown function, over-expression of NDRG2 has the potential to reduce or inhibit the growth of cancer cells by interfering directly or indirectly with the cell cycle. Although further clinical research is warranted, there are promising indications for using *NDRG2* expression as a diagnostic and prognostic biomarker in cancer.

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