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REVIEW

CHFR: a key checkpoint component implicated in a wide range of cancers

Sheru Sanbhnani · Foong May Yeong

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Abstract CHFR (Checkpoint with Forkhead-associated and RING finger domains) has been implicated in a checkpoint regulating entry into mitosis. However, the details underlying its roles and regulation are unclear due to conflicting lines of evidence supporting different notions of its functions. We provide here an overview of how CHFR is thought to contribute towards regulating mitotic entry and present possible explanations for contradictory observations published on the functions and regulation of CHFR. Furthermore, we survey key data showing correlations between promoter hypermethylation or down-regulation of *CHFR* and cancers, with a view on the likely reasons why different extents of correlations have been reported. Lastly, we explore the possibilities of exploiting *CHFR* promoter hypermethylation status in diagnostics and therapeutics for cancer patients. With keen interest currently focused on the association between hypermethylation of *CHFR* and cancers, details of how CHFR functions require further study to reveal how its absence might possibly contribute to tumorigenesis.

Keywords CHFR · Checkpoint · Microtubules · Entry into mitosis · Promoter hypermethylation · Cancers

Introduction

Mitosis is a crucial cellular event during which key processes of the cell division cycle culminate in the segregation of duplicated genetic material, thereby giving rise to two genetically identical daughter cells [1]. Errors that occur during mitosis could potentially lead to chromosomal instability or the propagation of mutations, both of which could ultimately lead to cellular transformation. However, checkpoints exist in eukaryotic cells that serve to delay cell cycle progression when the stability of the cells' genome is threatened. As such, checkpoints are important, especially in humans, to prevent the propagation of cells with corrupted genome that could potentially cause tumor formation [2].

For instance, mitotic entry is guarded by checkpoints that modulate the activities of key mitotic kinases in response to various genotoxic insults that could potentially compromise the fidelity of mitosis (Fig. 1a). At G2, the ATM/ATR-mediated DNA damage and replication checkpoints (reviewed in [3–5]) ensure that DNA integrity is intact and DNA replication is completed before allowing cells to enter mitosis. Also, a p38-mediated stress checkpoint acts to reverse chromosome condensation during antephase in response to a variety of stresses in the antephase checkpoint (reviewed in [6]).

Recently, CHFR (Checkpoint with forkhead-associated and RING finger domains) was identified as a mitotic checkpoint protein that delayed entry into metaphase in response to microtubule-targeting drugs. CHFR is a likely component of the antephase checkpoint that safeguards mitotic entry [6] but is not required for normal cell cycle progression [7]. In tumor cell lines such as colon cancer HCT116 cells in which *CHFR* is deleted or osteosarcoma U2OS cells in which *CHFR* is mutated and the gene product is non-functional, cell cycle progression into mitosis was not delayed upon exposure of these cells to stress agents such as the microtubule-depolymerizing drug Nocodazole (Noc) [7]. This was unlike cells with wild-type *CHFR* that delayed mitotic entry under the same

S. Sanbhnani · F. M. Yeong (✉)
Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, MD7, 8 Medical Drive, Singapore 117597, Singapore
e-mail: foong_may_yeong@nuhs.edu.sg

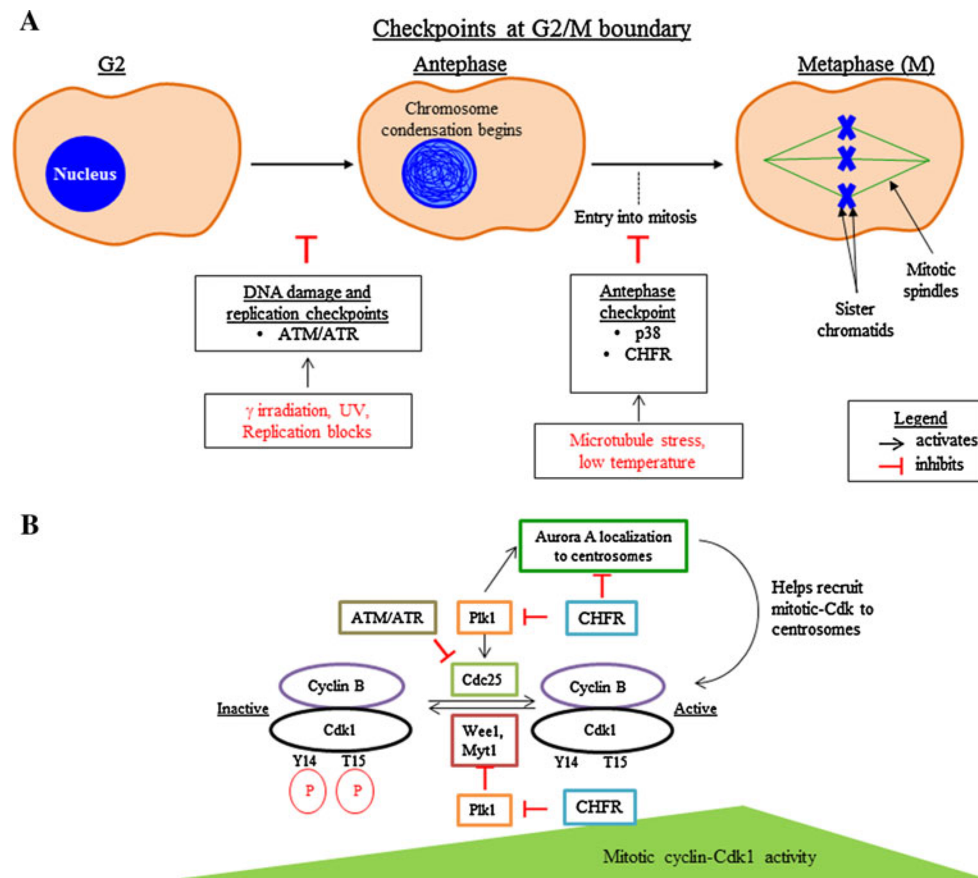

Fig. 1 Key checkpoints at the G2/M boundary. **a** Diagram showing a cell at the G2 phase progressing towards mitosis (metaphase). At this juncture, several key checkpoints exist such as the DNA replication and DNA damage checkpoints that act to inhibit Cdc25 through a signaling pathway involving ATR/ATM. If these checkpoints are not activated, the cell will enter antephase when chromosomes begin to condense. At this point, the antephase checkpoint will act to delay mitotic entry in a manner dependent upon p38 MAPK and CHFR. **b** Possible interactions between CHFR and key cell cycle regulators. CHFR acts to delay mitotic entry by inhibiting Plk1 and/or Aurora A. As a result of Plk1 and/or Aurora A inhibition, the mitotic cyclin-Cdk1 activity is down-regulated and cells fail to enter mitosis (see text for details). If the antephase checkpoint is not activated, the mitotic cyclin-Cdk1 activity will increase and promote mitotic entry of the cell. The cell condenses its chromosomes and eventually arrives at metaphase where chromosomes align at the metaphase plate. The mitotic cyclin-Cdk1 activity normally reaches a peak at metaphase

conditions. Restoration of CHFR function by transfection of wild-type *CHFR* into the cancer cell lines lacking functional CHFR re-established the mitotic entry checkpoint [7, 8].

Furthermore, treatment of *CHFR*-expressing PtK1 epithelial cells from rat kangaroo kidney with other microtubule-depolymerizing drugs such as colcemid at prophase resulted in a delay in mitotic entry in the cells [9]. In contrast, HeLa cells that do not express CHFR and U2OS cells that express a non-functional CHFR progressed into mitosis when treated similarly. Quite significantly, the presence of *CHFR* increased the viability of cells exposed to microtubule stress [7, 10], suggesting that CHFR plays a protective role in cells, perhaps by delaying cell cycle progression through blocking entry into mitosis in the presence of microtubule damage.

CHFR as a checkpoint component

The existence of key observations indicating a role for CHFR in a mitotic entry checkpoint notwithstanding, the exact molecular mechanisms underlying its function remain relatively unclear. However, its molecular structure (Fig. 2) has been functionally characterized to some degree with respect to its role in delaying cell cycle progression such as entry into mitosis.

Cell cycle regulation of CHFR during unstressed and stressed conditions

CHFR is ubiquitously expressed in normal human tissues and has an N-terminal forkhead-associated (FHA) domain, a central RING-finger (RF) domain and a C-terminal

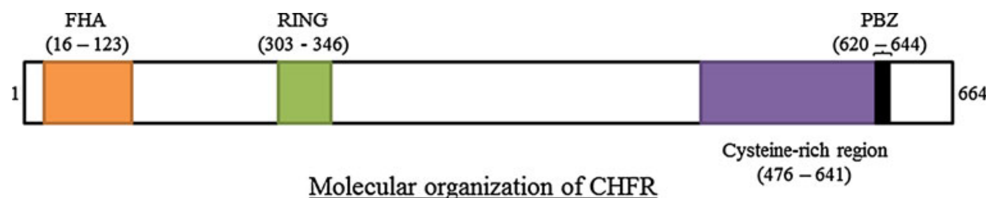

Fig. 2 The functional domains of CHFR. The forkhead-associated (FHA) domain is located at the N-terminal region of CHFR. This is followed by the RING-finger (RF) domain that has ubiquitination activity. CHFR also has a cysteine-rich region that is overlapping with the poly(ADP-ribose)-binding zinc finger (PBZ) motif near the C-terminal region. These domains play a role in the various functions of CHFR (see text for details)

cysteine-rich region [7]. The RF domain confers ubiquitin ligase (E3) activity on CHFR, as evident from in vitro and in vivo ubiquitination assays using full-length CHFR, RF domain-CHFR or various RF domain mutants [9–15]. The FHA domain, known to be a phosphopeptide-binding domain [16], appears to negatively influence the ubiquitination activity of CHFR [12].

More importantly, from other studies involving mutational analyses of CHFR and assays using methyl-ubiquitin that inhibited ubiquitination, it was revealed that the ubiquitination activity of CHFR is needed for its checkpoint function in G2/M transition [9–12]. For instance, transfection of *CHFR* into human cell lines such as colon carcinoma DLD1 cells or HeLa cells that do not express CHFR, or U2OS cells that express non-functional CHFR resulted in delayed mitotic entry when the cells were exposed to microtubule poisons [10, 11]. More importantly, the delayed mitotic entry was dependent upon the ubiquitination activity of CHFR. Also, in rat kangaroo PtK1 cells over-expressing a RF domain mutant of CHFR in which the ubiquitination activity is abolished, the cells failed to delay mitotic entry in the presence of colcemid [9].

Interestingly, the ubiquitination activity of CHFR appears also to destabilize CHFR in a cell cycle-regulated manner independently of stress. This notion is supported by the observation that the RF domain mutant of CHFR, but not the wild-type CHFR, remained stable over the cell division cycle when both forms were exogenously expressed in cells [10, 15]. When the putative auto-ubiquitination sites at lysine 384 and lysine 393 were mutated to alanines, the mutant CHFR was stabilized and caused a delay in mitotic entry [15]. Both groups [10, 15] further reported that CHFR levels peaked at G2/M in unperturbed cells. Based on their findings, Kim and coworkers [15] proposed that in unstressed conditions, the auto-ubiquitination of CHFR leads to the destruction of CHFR as cells reach G2/M, thereby allowing cells to enter mitosis during a normal cell division cycle. Upon exposure of the cells to microtubule poisons, CHFR level accumulates, consistent with its role in blocking mitotic entry during stress.

However, it is difficult to reconcile the authors' [15] idea that CHFR is destabilized by auto-ubiquitination prior to entry into mitosis during an unstressed cell cycle with the observations that the ubiquitination activity per se of CHFR appears to be needed for delaying mitotic entry during stress [9–12, 14, 15]. It is noteworthy that a mutant form of CHFR with an intact RF domain but in which its putative lysine residues targeted by auto-ubiquitination were replaced with alanines, was still capable of causing a delay in mitotic entry when expressed exogenously [15]. The ectopic expression of the RF mutant, however, failed to delay mitotic entry [10, 15], indicating that the RF domain is likely important for the mitotic entry checkpoint. Presumably, upon stabilization of CHFR during exposure to stress, the auto-ubiquitination activity that normally causes destruction of CHFR during an unperturbed cell division might be modulated in some way for ubiquitination of other substrates for its checkpoint function. A re-examination of how the auto-ubiquitination activity affects CHFR abundance during a normal versus a stressed cell division cycle in an experimental set-up similar to that described in [15] could be performed to clarify this point.

Contrary to the observations alluded to above that CHFR abundance changes during cell cycle, a study by Bothos and colleagues [11] revealed that HA-CHFR stably expressed in U2OS cells was phosphorylated rather than degraded in a cell cycle-dependent manner. Moreover, the authors observed that the phosphorylation of the exogenously expressed HA-CHFR in U2OS cells occurred in mitotic cells independently of microtubule poisons [11]. The authors proposed that the discrepancy between their results and that of the previous study [10] could be because antibodies used in the earlier study had failed to detect the phosphorylated CHFR, thereby leading to the authors' conclusion that CHFR abundance was regulated during the cell cycle [10]. This, however, does not explain the cell cycle-regulation of CHFR abundance observed in the study by Kim and colleagues [15] where anti-Flag antibodies were used for detection of Flag-CHFR in Western-blot analyses, as the detection of Flag-CHFR by the anti-Flag tag antibodies used was unlikely to be affected by phosphorylation. Rather, the differences could be that Bothos

and colleagues [11] had examined HA-CHFR that might have behaved differently than Flag-CHFR [15], in addition to the different cell lines used in these studies.

In a more revealing series of experiments, Burgess and colleagues [17] examined the endogenous CHFR in *Xenopus* egg extracts or *Xenopus* XL2 and XTC cell lines, and observed that the CHFR was ubiquitinated and phosphorylated but yet remained stable throughout the cell cycle. More significantly, upon over-expression of *CHFR* in the XL2 cells, the exogenously expressed CHFR was degraded in an ubiquitin- and proteasome-dependent manner, suggesting that cells are unable to tolerate a level of CHFR higher than the endogenous level. This observation could provide a possible explanation as to the inconsistencies among the various reports on CHFR ubiquitination and abundance as described above [10, 11, 15]. For instance, in the reports describing the cell cycle regulation of CHFR levels [10, 15], CHFR was expressed exogenously in various human cell lines, and the levels of CHFR in these cell lines might have been much higher than the endogenous levels. As a result, the CHFR was observed to be destabilized in the cells.

These observations further raise the questions as to whether the endogenous CHFR in fact behaves similarly to exogenously expressed CHFR in post-translational modifications and whether the endogenous CHFR could in fact initiate a delay in mitotic entry when cells are exposed to microtubule stress. In order to address these questions, studies should be performed using untransformed cells to observe the levels of endogenous CHFR over a cell division cycle. An apparent hurdle is the difficulty in detecting endogenous levels of CHFR [14] in certain cell lines such as U2OS with low levels of CHFR [7]. It could perhaps also be due to the destabilization of endogenous CHFR in the unstressed cells or the poor sensitivities of existing antibodies in detecting endogenous CHFR. As such, a better panel of antibodies needs to be generated. In combination with cell cycle synchronization methods (for example [15]), it would then be possible to establish in different model systems, whether endogenous CHFR levels fluctuate over a cell division cycle and if it is in fact phosphorylated and ubiquitinated. The effects of microtubule poisons on the stability of endogenous CHFR and if entry into mitosis were delayed upon up-regulation of CHFR could then be examined in greater details.

Possible modes of action of CHFR in delaying mitotic entry

While there exist discrepancies among the reports on the auto-ubiquitination, phosphorylation and proteolysis of CHFR, there are nonetheless considerable data showing that CHFR ubiquitinates and likely targets additional substrates for destruction by the 26S proteasome [18]. Such substrates have been proposed to include key regulators that control the activities of the mitotic cyclin-dependent kinases, Cdk1 or Cdk2 that promote mitotic events (reviewed by [19]).

Mitotic entry is tightly controlled at several levels. For instance, cells are unable to enter mitosis until the accumulation of mitotic cyclins such as cyclin B prior to mitosis (reviewed in [20]; Fig. 1b). Upon accumulation, cyclin B binds to and activates Cdk1, which then drives cells into mitosis. Given that the presence of non-destructible cyclin B in *Xenopus* extracts did not over-come the effects of recombinant CHFR in preventing mitotic entry [12], it is possible that the mitotic cyclins are not the direct target of CHFR.

Entry into mitosis is also normally regulated by the phosphorylation status of Cdks at the conserved threonine-14 (T-14) and tyrosine-14 (Y-15) residues [19]. Upon phosphorylation of these residues by the Myt1 and Wee1 kinases respectively, Cdk1 becomes inactive (Fig. 1b). The inhibitory phosphorylation on Cdk1 is relieved by the dephosphorylation of these residues through the action of one of the three isoforms of the Cdc25 phosphatases ([21]; Fig. 1b). The Myt1 and Wee1 kinases as well as the Cdc25 phosphatase are themselves under the regulation of another kinase known as the polo-like kinase (Plk1) [22]. Essentially, the down-regulation of both Myt1 and Wee1 by Plk1 allows Cdc25 to dephosphorylate T-14 and Y-15 on Cdk1. This, coupled with the activation of Cdc25C by Plk1, leads to an increase in the mitotic Cdk activities that would promote entry into mitosis (Fig. 1b).

Studies have shown that expression of *CHFR* causes the ubiquitination of Plk1 that results in its destruction, thereby causing a delay in mitotic entry. For instance, in in vitro assays using *Xenopus* extracts, the presence of recombinant CHFR led to the ubiquitination and destruction of the endogenous Plk1 [12]. Correspondingly, Cdc25C and Wee1 in the extracts remained unphosphorylated. With Cdc25C inactive and Wee1 active, Cdc2 was maintained in an inactive state and failed to drive entry into mitosis. A similar inverse relationship between CHFR levels and Plk1 levels was observed in HeLa cells exogenously expressing myc-CHFR [14] or Flag-CHFR [15]. However, given that these experiments were conducted in the absence of microtubule poisons, it would be important to examine if indeed such a pathway operates in the presence of microtubule stress to delay entry into mitosis.

It should be noted that Plk1 also interacts in a complex manner with Aurora A, a key kinase functioning during cell division, to regulate mitotic events including entry into mitosis (reviewed in [23, 24]). The phosphorylation of Plk1 by Aurora A leads to the activation of Plk1 in G2. In turn, Aurora A requires Plk1 activity for localization to the

centrosomes in late G2. Once localized, Aurora A promotes the recruitment of the mitotic cyclin, cyclin B1, to the centrosomes. Aurora A also phosphorylates and activates Cdc25B at the centrosomes, where Cdc25B triggers the activation of cyclin B1-Cdk1. Together with Plk1, Aurora A contributes towards the proper initiation of mitosis.

CHFR can also bind to Aurora A [13, 25, 26] via its cysteine-rich C-terminal domain [26]. The association between CHFR and Aurora A led to the ubiquitination of Aurora A and its destabilization in mouse embryo fibroblast cells in the presence of stress agents [26]. This is consistent with observations showing an inverse relationship between loss of CHFR function and Aurora A levels in *CHFR*$^{-/-}$ mouse embryonic fibroblasts (MEFs) [26], prostate [13] and breast [13, 25] cancer cell lines as well as colorectal cancer samples [27].

There are, however, other reports showing that the CHFR-mediated delay in mitotic entry in cells exposed to microtubule poisons does not lead to ubiquitin-mediated proteasomal degradation. For example, CHFR has been demonstrated in in vitro assays [11] to bind preferentially to the ubiquitin-conjugating enzyme Ubc13-Mms2 to form Lys63-linked poly-ubiquitin chains, which are involved in signaling rather than proteolysis during cellular stress [18]. Summers and coworkers found that endogenous Plk1 and Aurora A levels remained unchanged when CHFR was expressed in HCT116 cells treated with Noc [8]. Also in rat kangaroo PtK1 cells treated with microtubule-disrupting drugs, the CHFR-mediated delay in mitotic entry required only ubiquitination but not proteasomal degradation, as Plk1-GFP was observed to accumulate in the cells during the checkpoint activation [9]. Furthermore, the presence of proteasome inhibitors did not abrogate the CHFR-mediated checkpoint, further suggesting that mitotic entry was delayed independently of the degradation machinery.

It was also noted that in HCT116 cells, a delayed entry into mitosis following nocodazole treatment depended only on the FHA domain of CHFR [28]. Indeed, the authors noted that the RING finger domain needed for ubiquitination as well as the cysteine-rich region were dispensable. Fukuda and coworkers [28] explained that the discrepancy they observed as compared to previous studies [12, 26] could be due to different cell-types examined and epitope tags fused to CHFR. As the FHA domain is a phosphopeptide-binding module (reviewed in [16]), the observations suggest that CHFR likely plays a role in a signaling cascade that delays entry into mitosis. This could provide an explanation as to why Plk1 and Aurora A are not degraded during the CHFR-mediated checkpoint activation [8, 9], in contradiction to other reports alluded to above [12, 26].

The differences in the observations between studies showing CHFR-dependent degradation of Plk1 and/or Aurora A [12, 26], and studies showing the contrary [8, 9], could also be due to the distinct manner by which CHFR functions in distinct systems examined. For example, in the assays involving *Xenopus* extracts [12], the authors made use of recombinant human CHFR, which might function differently in such a hybrid assay system, thereby leading to the discrepancy observed as compared to the studies performed using cell lines [8]. Also, the studies conducted without exposing cells to stress [14, 15] should not be directly compared to cells that were [8, 9], as that might contribute to distinct modes of CHFR activity observed. Further analysis using approaches involving MEFs from appropriate knock-out mice to understand the relationships between endogenous CHFR and endogenous Plk1 and Aurora A levels might provide more ideas as to the molecular basis by which CHFR regulates mitotic entry under different conditions.

Another aspect of CHFR function that remains unclear is the mechanism by which CHFR senses the presence of microtubule stress. During a normal cell division, endogenous CHFR was observed to bind to the endogenous translationally controlled tumor protein (TCTP) as well as endogenous β-tubulin at the mitotic spindle [17]. It was proposed that disruption of the spindle due to exposure to microtubule poisons liberates CHFR from its interaction with TCTP and the spindle, which then enables CHFR to activate a signaling pathway that delays mitotic entry. Details of how this interaction contributes to CHFR function await further elucidation.

CHFR and its possible role in DNA damage checkpoint

Intriguingly, the checkpoint function of CHFR is not limited to microtubule stress. CHFR also has a role as part of a signaling cascade during DNA damage. In the presence of ionizing radiation, CHFR, together with ring finger protein 8 (Rnf8) that is an ubiquitin ligase [29], is involved in the ubiquitination of histones needed for signaling. For example, upon DNA damage induced by ionizing radiation, histone H2A and H2B are ubiquitinated by CHFR and Rnf8 [30].

The ubiquitination of histones does not lead to their destruction, but rather, results in the binding of a protein known as MOF-related gene on chromosome 15 (MRG15) to the ubiquitinated histone H2B. MRG15 then recruits histone acetylases such as MOF [31] and Tip60 [32, 33] to the chromatin. MOF and Tip60 acetylate histone H4 at lysine 16 (H4K16), which appears to be needed for the activation [31] of the key DNA damage checkpoint effector, ATM [3, 4]. ATM in turn, modifies the chromatin

surrounding DNA double strand breaks so as to enable the repair of the DNA breaks [34].

In addition to the FHA and RF domains, CHFR also carries a functional domain known as the poly(ADP-ribose)-binding zinc finger (PBZ) motif at the C-terminus that encompasses the cysteine-rich region [35]. Poly(ADP-ribosyl)ation is a process during which long chains of ADP-ribose units, linked by glycosidic ribose–ribose bonds, are added to substrates [36]. The cysteine residues in the PBZ domain of CHFR allow CHFR to bind to poly(ADP-ribose) (PAR). Although the PBZ motif was identified in a survey for proteins involved in DNA damage response and checkpoints, the PBZ motif in CHFR is needed for blocking transition into mitosis in the presence of microtubule stress [35].

The data thus far support the notion that CHFR plays an important role in checkpoint pathways that regulate cell cycle progression in the presence of stress. Presently, however, no consensus can be derived from the studies as to the molecular mechanisms by which CHFR delays entry into mitosis when cells are exposed to microtubule poisons. This is because of the inconsistent reports among the various studies (see above) on CHFR function and regulation. The discrepancies could have come about in part due to the distinct molecular environments that CHFR was examined in the various in vivo systems employed. Also, almost all the studies made use of tumor-derived cell lines. Since Plk1 and Aurora A are frequently over-expressed in certain cancers [24] and likely also in the cell-lines derived from them, this could have influenced the conclusions drawn as to the effects of CHFR on the stability of Plk1 and Aurora A observed.

In addition, in studies that have reported decreased mitotic indices as indications of delayed mitotic entry when cells exogenously expressing *CHFR* were exposed to microtubule stress, several of them failed to include unstressed controls (e.g., [15]). As such, any decrease in the mitotic index observed could be due to the mere expression of *CHFR* in these tumor cells in the absence of stress. Alternatively, exogenous expression of *CHFR* leading to reduced mitotic indices could have in fact delayed cells in other phases of the cell cycle. For instance, the decreased mitotic index observed in HeLa cells exogenously expressing Flag-CHFR was actually due to a cell cycle arrest in G1 [13], suggesting that CHFR might act in a manner more complex than thought. In several breast cancer cell lines, reduced mitotic index in the presence of CHFR did not necessarily translate to an increase in G2/M to G1 ratio after nocodazole treatment [37], further hinting that CHFR has roles other than in mitosis. Thus, synchronized experiments with appropriate controls using untransformed as well as tumor cells are needed to determine how CHFR imposes a cell cycle delay, and if its role is confined to G2/M. With a better basic characterization of CHFR, one can make better conclusions about how CHFR functions upon exposure of the cells to various stress conditions such as disruption of microtubules, DNA damage and perhaps other stress factors.

Regulation of CHFR

Presently, there are several studies providing hints as to how *CHFR* might be regulated at the transcriptional and post-translational levels. In one study, *CHFR* was predicted to be the target of the microRNA (miRNA) miR-26b, which was identified in a global screen for miRNAs involved in cellular proliferation [38]. miR-26b was found among several other miRNAs to be common in one human embryonic stem cell line and 4 colorectal cancer cell lines. Although this would suggest the regulation of *CHFR* at the transcript level, the molecular basis underlying such a possibility was not examined.

As alluded to above, CHFR abundance has been found to fluctuate during the cell division cycle due to auto-ubiquitination, albeit the observations were made in several mammalian cell lines over-expressing *CHFR* [10, 15] and not the endogenous CHFR. In unstressed cells, the phosphorylation of CHFR at G2 [11, 15] that corresponded with the auto-ubiquitination and degradation of CHFR prior to mitosis [15], likely enabled cells to enter mitosis. Consistent with this, in vitro phosphatase treatment of CHFR led to a reduction in auto-ubiquitination of CHFR [15]. Kim and colleagues [15] suggested that the cyclinA/Cdk2 complex might be the kinase that is responsible for CHFR phosphorylation, although no direct evidence was provided. They proposed that during exposure to microtubule stress, CHFR could be stabilized, thereby acting to delay mitotic entry [15]. These observations highlight the possibility that phosphorylation of CHFR regulates its function, though the pathway leading to CHFR phosphorylation remains uncharacterized.

Related to the ubiquitination activity of CHFR, a de-ubiquitination enzyme known as ubiquitin specific peptidase 7 (USP7) (reviewed in [39]) has been found to be a major binding partner of CHFR [40]. USP7 is important for regulating the stability of key cellular regulators such as the tumor suppressor p53, among several others [39]. In the context of CHFR, the co-transfection of His-USP7 and FLAG-CHFR in human embryonic kidney HEK293T cells resulted in the stabilization of FLAG-CHFR [40]. Moreover, in in vivo and in vitro assays, USP7 was able to cause de-ubiquitination and stabilization of CHFR. This confirmed the previous studies [10, 15] that showed the dependence of CHFR stability on its ubiquitination status. Further analysis of how the activity of USP7 is modulated during an unperturbed cell division cycle as well as during exposure of cells to stress will be important to provide a complete picture of the regulatory pathways affecting CHFR abundance in cells.

Another aspect of CHFR regulation is its localization to the nucleus [41]. In cells over-expressing GFP fusion of CHFR [17, 42], GFP-CHFR was localized specifically at nuclear foci that contain the promyelocytic leukemia protein (PML bodies). A FHA-domain deletion mutant of CHFR failed to co-localize with the PML bodies and exhibited dominant-negative effect on delaying entry into mitosis [42]. Also *PML*$^{-/-}$ MEFs were unable to respond to mitotic stress, indicating that the PML bodies are likely to be functionally important for CHFR to execute its checkpoint function. It would be necessary to understand the basis of how the localization of CHFR is regulated. Although studies on the dynamics of GFP-CHFR revealed that GFP-CHFR was highly mobile in the cytoplasm compared to GFP-CHFR localized to the PML bodies [42], the GFP-CHFR depended upon over-expression vectors that might not truly reflect the localization of the endogenous CHFR. The generation of anti-CHFR antibodies that are suitable for immuno-fluorescent staining (e.g., [17]) through selection from a panel of antibodies as well as optimization of the staining protocols should be useful for future studies to confirm if the endogenous CHFR does in fact co-localize with PML bodies.

More recently, it was reported that CHFR was negatively regulated by Stil (encoded by the SCL/TAL1 interrupting locus gene) in MEFs [43]. Stil is a cell cycle-regulated protein that accumulates in G2 [44, 45] and is able to cause auto-ubiquitination and destruction of CHFR [43]. The authors showed that targeting of CHFR for destruction via the proteasome by Stil results in passage of cells through mitosis. A point of note is the finding that *STIL* expression is correlated with cancer progression in various cancers [46, 47]. However, it is unknown if there is a corresponding decrease in CHFR levels in the cancers showing *STIL* expression and if that might possibly contribute in some way to the development of such cancers.

It remains to be seen precisely how *CHFR* expression, abundance, activity and localization are regulated. It is pertinent to dissect the molecular details of CHFR function and to derive a model of CHFR regulation during different conditions of cellular proliferation so that we might better understand the consequences of CHFR down-regulation in cancers (see below).

Clinical relevance of CHFR function

Down-regulation of *CHFR* in a range of cancers

The crucial role of CHFR in a checkpoint function is evidenced by reports showing the down-regulation of *CHFR* expression in various cancer cell lines including colorectal, hepatocellular, pancreatic, lung, myeloma, leukemia and head and neck, among others [7, 8, 48, 49]. In a comprehensive survey of cancer cell lines from various tissues by Toyota and colleagues [49], it was found that expression of *CHFR*, as determined by reverse transcription-PCR (RT-PCR), was down-regulated or lost in 33% of the cell lines examined.

In several of these cell lines, the down-regulation of *CHFR* is due to CpG hypermethylation-dependent silencing of the promoter [48, 49], presumably through the action of DNA methyltransferases such as Dnmt1 and Dnmt3b [49]. This relationship is likely to be important, as *DNMT*s are over-expressed in tumors corresponding to aberrant hypermethylation patterns in various genes needed for proper control of cellular processes such as cell division and gene transcription [50, 51]. More studies are needed to understand the mechanisms underlying the deregulated methylation of *CHFR* in these cells.

Other than reduced *CHFR* expression observed in established cancer cell lines indicating a possible contribution of *CHFR* deficiency towards cancers, *CHFR*$^{-/-}$ mice showed an increase in incidences of tumor formation [26]. This supports the notion that in normal cells, CHFR plays a critical role in regulating proper cell division, perhaps through delaying entry into mitosis via the down-regulation of Aurora A in addition to destabilizing Plk1 (see above). More notably, in addition to studies on cancer cell lines, numerous reports have documented *CHFR* promoter hypermethylation or down-regulation in various primary tumors. The studies are summarized in Table 1.

The association between hypermethylation of *CHFR* promoter and tumors was first reported in studies using samples from patients with primary lung cancer (Table 1). 19% (sample size of 37) of primary lung cancer samples examined showed hypermethylation at the *CHFR* promoter [48]. In a subsequent study on lung cancer focusing on non-small cell lung carcinoma (NSCLC) involving a sample size of 208 cases [52], *CHFR* hypermethylation was found to occur at a relatively low frequency of 14% of the cancer samples. Nonetheless, the authors reported a strong correlation between *CHFR* hypermethylation and smoking. Interestingly, in a separate study involving 157 NSCLC cases where CHFR levels were examined in paraffin-embedded tissue sections using immuno-histochemical (IHC) staining, the down-regulation of *CHFR* was found in 39% of the smoking-related NSCLC cases [53]. Relative to hypermethylation, the higher percentage of *CHFR* down-regulation suggested that in addition to promoter hypermethylation, perhaps other mechanisms might be involved in down-regulating *CHFR* levels in lung cancers. Overall, down-regulation of *CHFR* in lung cancer appears to be associated with poor prognosis [52, 53], although the molecular basis underlying the down-regulation of CHFR in NSCLC is presently unclear.

Table 1 Comprehensive list of studies examining CHFR down-regulation or hypermethylation in primary tumors and cancer cell lines

Cancer	Sample	Paired[a]	% methylation in normal tissue	Sample size[b]	% down-regulated[c]	% CHFR hyper-methylated	CHFR promoter hypermethylation correlated to downregulation[d]	% MSI/MSS/CIN[e]	Likely stage of CHFR inactivation	Correlation with lymph node metastasis	Direct correlation between CHFR hypermethylation and MLH1 methlyation[f]	Reference
Breast												
Breast cancer	Cell lines	NA	NA	24	50%	8%	No	ND	NA	NA	ND	[37]
Breast carcinoma	Primary tumors	No	ND	110	ND	1%	NA	1% MSI	ND	ND	ND	[94]
Breast cancer	Cell lines	IHMECs	0% down-regulated	22	41%	ND	NA	ND	ND	ND	ND	[95]
Ductal carcinoma in situ (DCIS)	Primary tumors	No	NA	33	ND	Rare	NA	ND	Early	ND	ND	[96]
Invasive ductal carcinoma (IDC)	Primary tumors	No	NA	33	ND	Rare	NA	ND	Not correlated with stage	ND	ND	[96]
Cervical												
Cervical carcinoma	Primary tumors	No	NA	24	ND	38%	NA	ND	Late (at tumorigenic stage)	ND	ND	[79]
Colorectal												
Colon adenocarcinomas	Primary tumors	Yes	29% (2/7), 22% (2/9)	30	ND	37%	NA	ND	ND	ND	ND	[97]
Colon cancer	Primary tumors	No	NA	25	80%	ND	NA	ND	ND	ND	ND	[54]
Colon cancer	Primary tumors	Yes	ND	22	ND	36%	NA	ND	ND	ND	ND	[54]
Colon cancer	Cell lines	No	NA	21		62%	Yes	62% CIN, 38% MIN	ND	ND	ND	[54]
Colon cancer	Cell lines	No	NA	7	71%	86%	NA	57% MSI, 43% CIN	NA	NA	ND	[56]
Colon cancer	Primary tumors	No	NA	62	ND	31%	NA	ND	Early	ND	Yes	[56]
Colon cancer	Primary tumors	Yes	ND	58	ND	26%	NA	ND	ND	ND	ND	[57]
Noninvasive colorectal cancer	Primary tumors	Yes	None	98	ND	26%	NA	ND	Early > advanced	No	ND	[59]
Colon cancer	Primary tumors	Yes	3%	108	ND	47%	NA	18% MLH1 methylation	ND	ND	ND	[58]
Colon cancer	Primary tumors	No	NA	888	32% (MI[g] > 4)	40%	NA	ND	ND	ND	ND	[55]
Colon cancer	Primary tumors	No	NA	71	ND	40.80%	NA	16.4% MSI + , 77.5% CIN+	Early	ND	Yes	[73]
Colon cancer	Primary tumors	6 normal tissues	Close to 0%	50	87.5	64%	Yes	80% MSS, 20%MSI	52% stage II, 48% stage III	No	ND	[27]

Table 1 continued

Cancer	Sample	Paired[a]	% methylation in normal tissue	Sample size[b]	% down-regulated[c]	% CHFR hyper-methylated	CHFR promoter hypermethylation correlated to downregulation[d]	% MSI/MSS/CIN[e]	Likely stage of CHFR inactivation	Correlation with lymph node metastasis	Direct correlation between CHFR hypermethylation and MLH1 methlyation[f]	Reference
Colon adenocarcinomas	Stage II and III tumors	No	9.1% in CHFR methylation-high	82	NA	54% (CHFR methyl-ation-high)	Yes	23% MSI	ND	Yes	No correlation found	[74]
Colon adenocarcinomas	Stage II and III tumors	No	NA	35	43%	NA	NA	23% MSI	ND	Yes	No correlation found	[74]
Endometrial												
Endometrial cancer	Primary tumors	Normal endo-metrium and atypical endometrial	0%	50	ND	12%	NA	ND	Early (Not correlated with tumor stage)	ND	ND	[86]
Endometrial cancer	Primary tumors	Yes	0%	63	ND	2%	NA	10% MLH1 methylation	ND	ND	ND	[58]
Esophageal												
Esophageal cancer	Primary tumors	Yes	None	38	ND	24%	NA	ND	Early (Correlated to all clinical stages)	No	ND	[59]
Esophageal cancer	Cell lines	NA	NA	15	26%	26%	Yes	ND	NA	NA	ND	[98]
Esophageal squamous cell carcinomas	Primary tumors	Yes	None	43	9%	16%	Yes	ND	Not correlated with stage	No	ND	[98]
Esophageal adenocarcinoma	Primary tumors	Yes	None	56	78.60%	21.40%	Yes	ND	ND	ND	ND	[67]
Gastric												
Gastric cancer	Cell lines	Normal stomach mucosa	0%	20	20%	20%	Yes	ND	ND	ND	ND	[83]
Gastric cancer	Primary tumors	Corresponding non-neoplastic gastric mucosa	5%	71	ND	35%	NA	ND	Not correlated with stage	No	ND	[63]
Gastric cancer	Primary tumors	Yes	8%	52	ND	35%	NA	35% MLH1 methylation, 17%	ND	ND	Yes	[62]
Gastric cancer	Cell lines	No	ND	9	33.00%	33%	Yes	ND	NA	NA		[65]

Table 1 continued

Cancer	Sample	Paired[a]	% methylation in normal tissue	Sample size[b]	% down-regulated[c]	% CHFR hyper-methylated	CHFR promoter hypermethylation correlated to downregulation[d]	% MSI/MSS/CIN[e]	Likely stage of CHFR inactivation	Correlation with lymph node metastasis	Direct correlation between CHFR hypermethylation and MLH1 methlyation[f]	Reference
Gastric cancer	Primary tumors	No	4%	46	ND	52%	Yes	ND	Not correlated with tumor stage or size	ND	ND	[65]
Gastric cancer	Primary tumors	Yes	None	41	ND	36.60%	NA	ND	ND	ND	ND	[99]
Gastric cancer	Primary tumors	Yes	None	12	25%	33%	Yes	ND	ND	ND	ND	[99]
Gastric cancer	Primary tumors	Yes	None	53	ND	30%	NA	ND	Correlated to all clinical stages	No	ND	[59]
Gastric cancer	Primary tumors	No	ND	174	33%	ND	NA	ND	ND	ND	ND	[60]
Gastric cancer	Primary tumors	No	NA	56	ND	41.10%	NA	ND	Not associated with tumor stage	No	ND	[100]
Gastric cancer	Primary tumors	Yes	Higher mRNA and protein expression	20	70%	45%	No	ND	Not correlated to tumor size	No	ND	[101]
Gastric cancer	Primary tumors	No	ND	69	55.07%	ND	NA	ND	ND	ND	ND	[101]
Gastric cancer	Primary tumors	Yes	None	25	ND	48%	NA	ND	ND	ND	ND	[102]
Gastric carcinoma	Primary tumors	Yes	10.20%	59	ND	33.90%	NA	17% MSI	Not correlated with tumor stage	No	ND	[66]
Gastric cancer	Primary tumors	Yes	11%	123	48%	41%	Yes	ND	Not correlated with pathological	No	ND	[64]
Gastric cancer	Primary tumors	Adjacent normal gastric mucosa	41.2% down-regulation	34	68%	59.00%	Yes	ND	NA	NA	ND	[64]
Gastric cancer	Primary tumors	Adjacent normal gastric mucosa	22.90%	70	ND	48.60%	NA	ND	Not correlated with pathological stage	Yes	ND	[64]
Gastric cancer	Primary tumors	Yes	30.60%	49	ND	63.30%	NA	ND	Not correlated to stage of tumor	No	ND	[80]
Advanced gastric cancer	Primary tumors	Yes	5.00%	20	ND	65.00%	NA	ND	ND	No	ND	[103]
Head and neck												
HNSCC	Primary tumors	No	ND	28	ND	25%	NA	ND	Correlated with stage IV	ND	ND	[104]

Table 1 continued

Cancer	Sample	Paired[a]	% methylation in normal tissue	Sample size[b]	% down-regulated[c]	% CHFR hyper-methylated	CHFR promoter hypermethylation correlated to downregulation[d]	% MSI/MSS/CIN[e]	Likely stage of CHFR inactivation	Correlation with lymph node metastasis	Direct correlation between CHFR hypermethylation and MLH1 methlyation[f]	Reference
Liver												
Hepatocellular cancer	Primary tumors	Noncancerous liver tissues	None	62	ND	35%	NA	ND	Late	ND	ND	[77]
Hepatocellular carcinoma	Primary tumors	No	ND	70	ND	43%	NA	ND	Late	ND	ND	[105]
Lung												
Lung cancer	Cell lines	Normal lung tissue	None	16	31%	31%	Yes	ND	NA	NA	ND	[48]
Lung cancer	Primary tumors	Normal lung tissue	None	37	ND	19%	NA	ND	ND	ND	ND	[48]
NSCLC	Primary tumors	Yes	0%	20	ND	10%	NA	ND	ND	ND	ND	[97]
NSCLC	Primary tumors	Yes	ND	22	45%	ND	NA	ND	ND	ND	ND	[106]
NSCLC	Primary tumors	Normal lung tissue	ND	157	39.40%	ND	NA	ND	Late	No	ND	[53]
NSCLC	Primary tumors	Normal lung tissue	ND	20	30%	15%	Yes	ND	ND	ND	ND	[53]
NSCLC	Primary tumors	Yes	0%	10	ND	20%	NA	ND	ND	ND	ND	[107]
NSCLC	Primary tumors	No	ND	208	ND	14%	NA	ND	Late	Yes	ND	[52]
NPC												
Nasopharyngeal carcinoma	Cell lines	Immortalized NPC cell lines	None	8	100%	100%	Yes	ND	NA	NA	ND	[108]
Nasopharyngeal carcinoma	Primary tumors	Non-neoplastic nasopharynx tissue	None	36	ND	61.10%	NA	ND	ND	No	ND	[108]
Nasopharyngeal carcinoma	Nasopharyngeal brushings, NPC paraffin tissues	No	NA	53	ND	58.50%	NA	ND	ND	ND	ND	[109]
OSCC												
Oral squamous cell carcinoma	Cell lines	NA	NA	6	16.70%	33.30%	In cases with complete methylation only	ND	NA	NA	ND	[78]
Oral squamous cell carcinoma	Primary tumors	No	NA	49	ND	34.70%	NA	ND	Advanced	No	ND	[78]
Oral squamous cell carcinoma	Primary tumors	Yes	7.70%	13	ND	46.10%	NA	ND	ND	ND	ND	[78]

Table 1 continued

Cancer	Sample	Paired[a]	% methylation in normal tissue	Sample size[b]	% down-regulated[c]	% CHFR hyper-methylated	CHFR promoter hypermethylation correlated to downregulation[d]	% MSI/MSS/CIN[e]	Likely stage of CHFR inactivation	Correlation with lymph node metastasis	Direct correlation between CHFR hypermethylation and MLH1 methlyation[f]	Reference
PNST												
Malignant peripheral nerve sheath	Primary and recurrent tumors	No	ND	96	66%	ND	NA	ND	Not correlated to tumor size	ND	ND	[110]
Malignant peripheral nerve sheath	Primary and recurrent tumors	No	ND	10	80%	ND	NA	80% CIN	ND	ND	ND	[110]
Malignant peripheral nerve sheath tumor	Primary and recurrent tumors	Yes	0%	7	43%	ND	NA	ND	ND	ND	ND	[110]
Lymphoma												
Cutaneous T-cell lymphoma	Primary tumors	7 CD4 + T cell samples from healthy volunteers and three skin biopsy specimens from patients with dermatoses	None	28		19%	NA	0% MLH1 methylation	ND	ND	ND	[111]
Rectal cancer												
Rectal adenocarcinoma	Primary tumors	35 tissues adjacent to tumor and 19 normal controls	14.28% for adjacent tissue, 5.26% for normal	51	ND	45	NA	ND	Generally inversely correlated with Duke's stage	ND	ND	[112]

Various studies have been summarized to highlight key features examined such as cancer type, sample size, percentage down-regulation of *CHFR*, percentage promoter hypermethylation of *CHFR* and correlation between promoter hypermethylation of *MLH1* and *CHFR*

[a] Refers to whether normal tissues were paired with tumor samples for the analysis

[b] Green highlight refers to studies with sample sizes > 40

[c] Refers to percentage decrease in protein or mRNA levels

[d] Refers to whether the samples were analyzed for promoter methylation AND mRNA levels, protein levels by Western-blot analysis or IHC

[e] Refers to microsatellite instability (MSI), microsatellite stable (MSS) or chromosome instability (CIN) status of the samples and if there is a correlation to CHF R promoter hypermethylation

[f] Refers to whether the samples were analyzed for correlation between CHFR down-regulation or promoter methylation, and promoter methylation of MLH1

[g] MI refers to methylation index

To date, yet other studies have shown correlations between *CHFR* down-regulation and other cancer types. However, wide ranges in the frequencies of *CHFR* down-regulation in various cancers have been reported, likely due to the differences in sample sizes of clinical cases studied (Table 1). For example, a study on colon cancer involved a sample size as small as 22 (11 paired normal and tumor samples) [54] while another one as large as 888 (tumor samples only) [55]. In a few studies on colon cancer involving sample sizes of at least 50 cases, however, frequencies of *CHFR* promoter hypermethylation or down-regulation ranging between 26 and 40% have been noted ([3, 55–59]; Table 1). In gastric cancers, IHC examination of 174 gastric cancer samples revealed that 33% of the samples had negative CHFR staining [60], indicating a loss of *CHFR* expression. Other studies on gastric cancers with at least 50 cases (Table 1) showed reduced *CHFR* expression in about 34–49% of the samples.

However, as no normal tissue controls was included in several of these studies (e.g., [3, 55–57, 59, 60]), it might be that the percentages of *CHFR* down-regulation shown constituted an over-estimation. Indeed, in a few studies on colon [58] and gastric cancers [61–66] in which normal tissues had been analyzed together with the cancer tissues, the *CHFR* promoter was found to be methylated even in the normal tissues. Nonetheless, the levels of promoter hypermethylation in the cancer tissues were generally higher than that in normal tissues in these studies. This indicates that the down-regulation of *CHFR* might indeed be of functional significance in cancers, although it is not possible to conclude if the contribution of *CHFR* down-regulation to the cancers was similar in the different samples.

Promoter hypermethylation is but one mechanism by which reduced *CHFR* expression occurs in cancers. Indeed, Soutto and coworkers noted in their study on esophageal adenocarcinoma that 78.6% of 56 cases had reduced *CHFR* mRNA levels although promoter hypermethylation was detected in only 21.4% of these 56 cases [67]. The authors found that *CHFR* gene copy number was reduced in the tumor tissues but not in adjacent normal tissues, thereby accounting for the higher numbers of tumors showing *CHFR* down-regulation than promoter hypermethylation. Consistent with this finding is the location of *CHFR* at 12q24, where frequent loss of genomic copy number has previously been reported [68, 69].

Possible synergy between *CHFR* and *MLH1* down-regulation with microsatellite instability (MSI)

The varying degrees of association found between *CHFR* down-regulation and a particular cancer type (Table 1) could further be due to other genetic differences in the samples. Several studies indicated that possible genetic interactions between *CHFR* and the DNA mis-match repair system might contribute towards cancers and hence account for the variations in the extent of correlation reported between *CHFR* down-regulation and cancers. *hMLH1*, the homologue of the mis-match repair protein MutL in bacteria [70], has been documented to be down-regulated by promoter hypermethylation (reviewed in [71, 72]) in several cancer types such as gastric and colon cancers.

The correlation between hypermethylation of the *MLH1* promoter and incidences of *CHFR* promoter hypermethylation in primary colon cancers [56, 73] and gastric cancers [62] appears to be statistically significant (Table 1). Although these studies [56, 73] did not attempt to correlate promoter hypermethylation of both *CHFR* and *MLH1* with a decrease in CHFR and MLH1 protein levels, yet other studies have previously established an inverse relationship between *CHFR* promoter hypermethylation and levels of CHFR protein ([64, 74]; (Table 1)). Specifically, Tanaka and coworkers [74] found that *CHFR* expression level was inversely correlated with the degree of promoter hypermethylation in colon cancer, with undetectable or weak CHFR protein levels being associated with methylation index (MI) above 30% and medium or high expression levels being associated with MI below 30%.

The possible synergistic effect of the down-regulation of both CHFR and MLH1 on tumor formation was tested in an experimental mouse system [27]. *CHFR*$^{-/-}$ *MLH1*$^{-/-}$ mice showed a heightened incidence of tumor development as compared to wild-type, *CHFR*$^{-/-}$ *MLH1*$^{+/+}$ and *CHFR*$^{+/+}$ *MLH1*$^{-/-}$ mice. Generally, tumors developed very early in the *CHFR*$^{-/-}$*MLH1*$^{-/-}$ mice compared with the wild-type or heterozygous mice. Further analysis revealed that 1 out of 6 *CHFR*$^{+/+}$ *MLH1*$^{-/-}$ lymphomas displayed a dramatic reduction in *CHFR* expression relative to those in *CHFR*$^{+/+}$ *MLH1*$^{+/-}$ mice [27]. Using mouse embryonic fibroblast (MEF) cells from these knock-out mice, it would be important to determine the molecular basis of how the two gene products function to ensure proper genomic integrity during cell division so that we might understand how the loss of both genes leads to synergistic effects on tumorigenesis. The correlation between the loss-of-function of *MLH1* and *CHFR* down-regulation also warrants further study, as that might shed light on the basis and implications of the association between hypermethylation of *MLH1* and *CHFR* promoters observed in cancer samples. This might be investigated in detail using MEFs as well as the knock-out mice to clarify issues such as the progression of *CHFR* down-regulation in *MLH1*$^{-/-}$ MEFs or mice.

The down-regulation of *MLH1* is strongly correlated with microsatellite instability (MSI) in cancers including

colorectal and ovarian cancers (reviewed in [75, 76]). The correlation between *CHFR* hypermethylation and MSI though, remains unclear. In gastric cancer, *CHFR* promoter hypermethylation was strongly correlated with MSI in one study [66] while analysis of colon cancer microarray data by Fu and colleagues revealed that *CHFR* mRNA expression was significantly lower in MSI tumors than in microsatellite stable (MSS) tumors [27]. In contrast to this, a separate study by Derks and coworkers revealed no statistically significant correlation between *CHFR* hypermethylation and MSI in colorectal cancer, although *CHFR* hypermethylation occurred at a higher frequency in MSI-positive cancers [73]. Homma and coworkers also failed to find a correlation between *CHFR* hypermethylation and MSI-high phenotype in gastric cancer, despite observing a significant correlation with *MLH1* hypermethylation [62].

Adding to the complicated relationship between *CHFR* down-regulation and MSI, the hypermethylation of *CHFR* promoter has also been found in colorectal and gastric cancers displaying the MSS phenotype and chromosomal instability (CIN) [54, 62, 66, 73]. In contrast, Derks and colleagues found that *CHFR* hypermethylation is significantly correlated with the CIN-negative phenotype [73].

The examination of clinical samples and experiments with mouse models thus far support the notion that *CHFR* down-regulation is closely implicated in cancers. The findings also imply that *CHFR* is likely to synergize with that of *MLH1* in cancer development. However, in spite of links observed between hypermethylation of both *CHFR* and *MLH1* promoters, *CHFR* down-regulation is associated with MSI, MSS and CIN to varying degrees in different cancers. This is unlike *MLH1* that shows a tight correlation with MSI [75, 76]. The lack of distinct and specific association between decreased CHFR function and MSI would mean that a loss of CHFR function likely plays a minor role (if at all) in the key events leading to MSI. It should also be noted that *CHFR* promoter hypermethylation could occur in the absence of *MLH1* hypermethylation and vice versa [54, 60], indicating that the combined deficiencies of *CHFR* and *MLH1* contribute only to a subset of cancers involving down-regulation of either genes.

Even so, there is a need to better understand the clinical relevance of *CHFR* down-regulation with studies that include paired normal and tumor samples, and better characterization of the genetic as well as clinico-pathological background of the patients. At the mechanistic level, it would be important to study how the various modes of down-regulation of *CHFR* might occur and at a more challenging level, how this might contribute to tumor formation or progression.

Stage of CHFR inactivation in tumor progression

Attempts have also been made to correlate the inactivation of *CHFR* to different stages of various cancers, though no clear pattern has emerged as to when *CHFR* inactivation takes place normally during tumor progression. For instance, in colorectal cancer, the inactivation of *CHFR* was found to have occurred at an early stage [56, 59, 73] (Table 1), while a study on 38 esophageal cancer specimens with corresponding non-malignant tissues showed *CHFR* hypermethylation independent of cancer stages [59]. In hepatocellular cancer [77], NSCLC [52] and oral squamous cell carcinoma (OSCC) [78], *CHFR* inactivation has been observed occurring at a late stage (Table 1). It is likely that the hypermethylation of *CHFR* promoter is a late event during tumorigenesis as illustrated in an in vitro system of HPV-induced cervical carcinogenesis [79].

It has been suggested that early hypermethylation of a gene is likely to be responsible for transformation, but later hypermethylation could simply be the result of transformation [90]. Thus, as *CHFR* promoter hypermethylation has been observed in cancer samples in various stages, the implication is that *CHFR* promoter hypermethylation might not have any functional consequence in the early events of tumorigenesis in some instances. As such, hypermethylation of the *CHFR* promoter is perhaps only a contributing factor in the progression of tumor formation. It remains to be seen the consequences of *CHFR* down-regulation in contributing towards tumor progression, though such studies are likely to be complicated by confounding issues such as genetic background (see above).

Perspectives

Use of *CHFR* status as diagnostic or prognostic markers

The findings showing that *CHFR* promoter hypermethylation is correlated with certain tumor incidences highlight the question as to whether *CHFR* methylation status could have practical purposes. For instance, given the tight association between *CHFR* hypermethylation and poor outcomes in patients with smoking-related NSCLC [53], the status of *CHFR* promoter methylation might potentially serve as a prognostic marker for smoking-related NSCLC. *CHFR* promoter methylation status might also be a useful marker for colorectal cancers. For example, in a study on colorectal and endometrial carcinomas that included a panel of 23 genes studied such as *hMLH1*, *CHFR* promoter hypermethylation correlated with colorectal but not endometrial carcinomas [58]. Interestingly, *CHFR* promoter hypermethylation was also better-associated with sporadic rather than familiar colorectal carcinomas.

In gastric cancers, a study was conducted that examined the suitability of hypermethylation as a diagnostic marker for micrometastasis [80]. Promoters of *CHFR*, among others such as *hMLH1*, were examined in 49 primary gastric tissues with matched non-cancerous gastric mucosa and regional lymph nodes. The hypermethylation status of these promoters in the regional lymph nodes was taken as a means of establishing the extent of micrometastasis in gastric cancers. Interestingly, the hypermethylation of *CHFR* that occurred in the regional lymph nodes correlated with that seen in the cancer tissues. As lymph node micrometastasis might a useful prognostic marker for recurrence of gastric cancers in patients [81], *CHFR* hypermethylation status could serve as a practical molecular tool. Perhaps, by its inclusion into the list of surrogate markers for hypermethylation [82], *CHFR* methylation status might help better inform the diagnosis or prognosis of specific cancers, given the possible contribution that loss of *CHFR* function might make towards cancer progression (see above).

Treatment of cancers based on *CHFR* hypermethylation status

Another potential translational application of the findings that *CHFR* is down-regulated in cancers could be the targeting of *CHFR* in cancer therapy. Indeed, it was found that in gastric cancer cell lines where aberrant *CHFR* promoter hypermethylation was present, the cells were sensitive towards docetaxel or paclitaxel (both belonging to the microtubule inhibitors known as taxanes) [83]. In these cell lines, the taxane-sensitivity can be abolished with exposure to 5-azacytidine, which is an inhibitor of DNA methyltransferase. Similar results were obtained in a study on several endometrial cancer cell lines exposed to paclitaxel [84]. In yet another study on cervical squamous cell carcinoma-derived cell lines where *CHFR* promoter was methylated, the cells were sensitive specifically towards taxanes but not to other anti-cancer agents such as cisplatin and doxorubicin [85]. The sensitivity towards taxane was reversed upon treatment of the cells with 5-azacytidine. Similar observations were made also in cell lines derived from endometrial cancer [86]. Furthermore, in OSCC cell lines in which *CHFR* is down-regulated, the cells were sensitive to exposure to docetaxel [87].

The significance of these observations is seen from the treatment of patients with gastric cancers who responded well to docetaxel [65]. These patients showed *CHFR* hypermethylation compared to those that did not respond well to the same treatment, indicating that the status of *CHFR* could be an indicator of responsiveness towards microtubule inhibitors in the treatment of gastric cancers. A similar correlation between *CHFR* promoter hypermethylation and sensitivity was found in NSCLC [88].

As mentioned above, a lowered *CHFR* expression was found to be associated with smoking-related NSCLC and poor prognosis [52, 53, 88]. In a separate study, it was found that serum from patients with NSCLC could be a practical source from which *CHFR* hypermethylation could be determined [89]. More importantly, the study indicated that patients with un-methylated *CHFR* showed longer survival when treated with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib. Taken together, these studies provide alternate perspectives in the treatment of NSCLC with respect to *CHFR* hypermethylation status.

In conclusion, more studies are needed to understand what the exact molecular role of CHFR is as a checkpoint component monitoring various stress factors that would affect cell cycle progression. Also, how its functions are regulated during cellular proliferation under normal versus stressed conditions need to be determined in greater details. The clarification of these issues is important, as it would help provide some explanation as to why the deregulation of *CHFR* expression is associated with certain cancers. It would further be critical to properly establish if *CHFR* hypermethylation contributes to the process of cancer formation or if it is merely a passenger DNA hypermethylation event [90]. That *CHFR* down-regulation or promoter hypermethylation has been found in clinical samples of different cancer stages supports the latter idea (see above). It is nonetheless important to address the issues of how aberrant *CHFR* promoter hypermethylation arises during tumorigenesis and how a deficiency in CHFR function contributes to cancer progression in combination with down-regulation of other genes such as *MLH1*. Given the close association between abnormalities in hypermethylation and cancers in general [91, 92] as well as *CHFR* hypermethylation status and certain cancers (see above), *CHFR* might further be useful when used in combination with other markers as a diagnostic tool or as a therapeutic target in the move towards personalized cancer therapy [93].

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Glossary of terms

- Antephase—refers to the time in late G2 phase when signs of chromosome condensation first become visible until commitment to mitosis [6].

- ATM—refers to Ataxia telangiectasia mutated, which is a key checkpoint kinase that plays a role the activation of the DNA damage checkpoint (reviewed in [3, 4]). It is important for a cell to respond to radiation-induced double-strand breaks by eliciting cell cycle delay and repair of the DNA breaks.
- ATR—refers to Ataxia telangiectasia mutated- and Rad3-related that is another major component of the DNA damage and replication checkpoints (reviewed in [5]). ATR is activated in the presence of DNA damage and replication blocks. Similar to ATM, activation of ATR leads to triggering of cell cycle delay and repair of the DNA damage.
- CIN—refers to Chromosome Instability. CIN relates to a persistent high rate of chromosome loss or gain due to mis-segregation of chromosomes during cell division (reviewed in [113]). This usually leads to aneuploidy in the resulting cells and is thought to contribute to tumorigenesis.
- DNA mis-match repair system—The system consists of proteins that are involved in repair of errors made due to mis-incorporation of nucleotides during the process DNA replication (reviewed in [70]). Such activities help to keep mutation rates low in dividing cells.
- FHA domain—Fork-head associated domain refers to the phosphothreonine-binding domain that is found in a range of proteins with diverse functions [114]. The domain functions essentially to monitor the status phosphorylation of specific threonine residues found in target proteins. The FHA domain is quite common in proteins that are involved in DNA damage response pathways.
- Microsatellite—refers to tandem mono-, di-, tri- and tetranucleotide repeats (e.g., A_n or (CA) that are distributed in our genome [115]. The correction of errors in the microsatellite depends upon the DNA mis-match repair system [70].
- Mitotic index—This refers to the fraction of the total number of cells examined that show condensed chromosomes [7, 8].
- MSI—Microsatellite instability refers to the errors associated with microsatellites that fail to be (reviewed in [71, 75]).
- MSS—Microsatellite stable refers to the absence of MSI [71].
- RING-finger (RF) domain—RING stands for Really Interesting New Gene. The RING-finger domain is a type of Zinc Finger domain that is a small motif that folds around one or more zinc ions [116]. The RING-finger domain is found in ubiquitin ligases such as the E3 ligases that are important for ubiquitin-mediated destruction of proteins.
- Ubiquitin and E3 ubiquitin ligase—Ubiquitin is a ubiquitous polypeptide with 76-amino acid residues (reviewed in [18]). It is activated by ATP by the action of a ubiquitin-activating enzyme known as E1. The ubiquitin is then transferred to a ubiquitin-conjugating enzyme known as E2. The E3 ubiquitin ligase is needed to help the E2 enzyme attach the ubiquitin to target proteins. Ubiquitin is attached to lysine residues on target proteins. If several ubiquitins are added to a single lysyl residue on the target protein, the target protein is referred to as poly-ubiquitinated. In some instances, ubiquitin is added to several distinct lysine residues on a target protein. In such cases, it is referred to as multi-ubiquitination.

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