Emerging evidence for \textit{CHFR} as a cancer biomarker: from tumor biology to precision medicine

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Abstract Novel insights in the biology of cancer have switched the paradigm of a “one-size-fits-all” cancer treatment to an individualized biology-driven treatment approach. In recent years, a diversity of biomarkers and targeted therapies has been discovered. Although these examples accentuate the promise of personalized cancer treatment, for most cancers and cancer subgroups no biomarkers and effective targeted therapy are available. The great majority of patients still receive unselected standard therapies with no use of their individual molecular characteristics. Better knowledge about the underlying tumor biology will lead the way toward personalized cancer treatment. In this review, we summarize the evidence for a promising cancer biomarker: checkpoint with forkhead and ring finger domains (\textit{CHFR}). \textit{CHFR} is a mitotic checkpoint and tumor suppressor gene, which is inactivated in a diverse group of solid malignancies, mostly by promoter CpG island methylation. \textit{CHFR} inactivation has shown to be an indicator of poor prognosis and sensitivity to taxane-based chemotherapy. Here we summarize the current knowledge of altered \textit{CHFR} expression in cancer, the impact on tumor biology and implications for personalized cancer treatment.

Keywords \textit{CHFR} promoter methylation · Predictive biomarker · Taxane sensitivity

1 Introduction

Over the last 20 years, there has been a revolution in the perspective of cancer treatment. Improvement of molecular profiling techniques such as next generation sequencing and whole genome methylation analysis made it possible to compare thousands of molecules simultaneously with high accuracy and speed. These studies have allowed novel and meaningful insights in the biology of cancer. Lung, breast, colorectal and many other cancers have shown to be heterogeneous...
diseases, which develop through specific molecular alterations that influence the clinical presentation, prognosis and response to therapy [1]. The diversity of molecular background and resultant biological behavior can be harnessed into an individualized biology-driven treatment, instead of the present “one-size-fits-all” approach. For some cancers, personalized cancer treatment is already implemented in daily practice.

In breast cancer treatment, for instance, it is now standard to test and target increased human epidermal growth factor receptor 2 (HER2) with both monoclonal antibodies such as trastuzumab or small molecule inhibitors such as lapatinib [2]. In non-small cell lung cancer (NSCLC) patients, testing for mutations in EGFR and KRAS, and EML4-anaplastic lymphoma kinase (ALK) gene rearrangements to select appropriately targeted therapy occurs on a routine basis. Mutations in the kinase domain of EGFR have shown to be a strong predictor of response to EGFR tyrosine kinase inhibitors (TKIs) erlotinib and gefitinib [3]. These patients respond better to EGFR TKIs than to chemotherapeutic agents carboplatin/paclitaxel, reflected by a significantly improved progression-free survival [4]. Furthermore, patients with NSCLC harboring the ALK rearrangement, which occurs in about 7% of NSCLCs, benefit from ALK inhibitor crizotinib [5]. A recent prospective randomized phase III study compared crizotinib therapy to chemotherapy, pemetrexed or docetaxel, in 347 locally advanced or metastatic ALK-positive lung cancers, and clearly showed that crizotinib therapy is associated with a higher response rate 65% (95% CI, 58 to 72) versus 20% (95% CI, 14 to 26) (\( P<0.001 \)) but also an improved quality of life compared to chemotherapy. The relatively low incidence of EGFR and ALK aberrations in non-Asian patients, however, account for the fact that ~87% of patients with NSCLC still receive conventional chemotherapy with no suitable biomarkers for therapy selection. The same accounts for women with triple-negative breast cancer who do not benefit from anti-hormonal therapy or trastuzumab and for whom effective treatment is limited [6].

Other examples of useful biomarkers are KRAS mutation testing to predict benefit from monoclonal antibodies against EGFR, cetuximab and panitumumab, in metastatic colon cancer [7] and BRAF V600E mutation analysis in metastatic melanoma in order to predict responsiveness to BRAF inhibitors such as vemurafenib [8]. Salient to this review, testing for promoter CpG island methylation of DNA repair gene O6-methylguanine-DNA methyltransferase, MGMT, guides the clinical management of glioblastoma. MGMT is able to reverse the damage acquired by alkylating agents and therefore promotes methylation, and subsequent silencing of MGMT is associated with increased progression-free and overall survival after therapy with alkylating agents such as temozolomide [9, 10].

Although these examples display the promise of personalized cancer treatment and more biomarkers are being discovered, work is still in progress. For most cancers and cancer subgroups, no biomarkers and effective targeted therapy are available and therefore the great majority of patients still receive standard therapies with no individualization based on their tumor’s molecular characteristics.

In this review, we highlight a promising novel biomarker for which multiple lines of evidence are emerging: checkpoint with FHA and ring finger domains (CHFR). CHFR is a mitotic checkpoint- and tumor suppressor gene and is inactivated in a diverse number of solid malignancies. CHFR is most frequently inactivated by promoter CpG island methylation and has shown to be a marker of poor prognosis and increased sensitivity to treatment with taxanes. Here we summarize literature on the relevance of altered CHFR expression in cancer.

2 CHFR: an important regulator of cell cycle progression

CHFR is an early mitotic checkpoint gene that functions as a key player in controlling chromosomal integrity [11].

CHFR is expressed in the cytoplasm of all normal tissues and accumulates in the nucleus in response to microtubule poisoning or radiation damaging stress. After localization into the nucleus, CHFR becomes phosphorylated by protein kinase B (PKB/AKT), a member of the PI3K signaling pathway [12]. The nuclear distribution, mobility and function of CHFR are dependent upon interaction with promyelocytic leukemia protein (PML) bodies [13, 14]. CHFR expression levels fluctuate greatly during different stages of the cell cycle. Microtubule stress will lead to an elevation of CHFR expression levels and a mitotic arrest. To the contrary, auto-ubiquitination activity and degradation of CHFR, which is stimulated by AKT, are a prerequisite for mitotic entry [15]. Thereby, CHFR controls cell cycle progression at the G2/M transition as well.

It is not known how CHFR senses microtubule stress, but it has been shown that CHFR localizes to the mitotic spindle by an interaction with TCTP, a protein involved in microtubule stabilization and β-tubulin [16]. Disruption of the spindle causes CHFR to delocalize from TCTP and the mitotic spindle, which will enable the activation of signaling pathways and ultimately delay cell cycle progression [17].

These signaling pathways prevent entry into mitosis by inhibiting the activation of Cdc25 phosphatases that are able to activate the cyclin B1-Cdk1 kinase.

CHFR is able to influence the mitotic checkpoint by a proteasomal-dependent and a proteasomal-independent mechanism (Fig. 1).

CHFR was first described to function as an E3 ubiquitin ligase, which ubiquitinates and targets proteins for degradation by the 26S proteasome [15, 18]. One of the target proteins is polo-like kinase 1 (PLK1). PLK1 is a serine/threonine
kinase that is involved in the phosphorylation of Cdc25, thereby regulating cyclin B1-cdk activity. PLK1 phosphorylation and activation are established by another kinase, Aurora A [19–22]. By ubiquitination and degradation of both PLK1 and Aurora A, CHFR is able to inhibit the formation of the cyclin B1-Cdk complex and thereby promote cell cycle arrest [20, 23]. Although in vitro data are appealing, evidence that CHFR targets PLK1 for degradation in vivo as well is weak. There are conflicting studies that did not observe a decrease in PLK1 and Aurora A protein expression in response to microtubule poison [24–26]. Differences in study design and molecular environment make it difficult to compare results and therefore more studies are needed to clarify this inconsistency.

Other targets for ubiquitination and protein degradation by CHFR are chromokinesine protein Kif22 [27], histone deacetylase HDAC1 [28] and poly(ADP-ribose) 1 PARP1 [29]. Kif22 plays a role in the organization of spindle microtubules and chromosome movement and regulation of Kif22 activity by CHFR is important for maintaining chromosomal stability [27]. HDAC1 is a histone deacetylase that is able to inhibit the expression of cell cycle genes such as p21. By ubiquitination of HDAC1, CHFR is able to reverse HDAC1-induced repression of p21 and thereby restore the p21-G1 checkpoint [28, 30, 31]. Interestingly, CHFR was also shown to inhibit invasiveness and metastatic potential caused by HDAC1 expression by the regulation of metastasis suppressors, KAI1 and E-cadherin [28].

PARP1 plays a role in the DNA damage response and is involved in the recruitment of CHFR to DNA damage sites immediately after DNA damage has occurred [29, 32]. CHFR then participates in a cascade of protein ubiquitination. One of the proteins that becomes ubiquitinated and degraded is PARP-1 itself. Thereby, CHFR is able to detach PARP-1 from the chromatin, which is an important step in the DNA damage repair response [29].

CHFR binds to PARP-1 via the RAR-binding zinc finger domain, which is situated in the cysteine region of CHFR. As the name illustrates, CHFR contains a N-terminal FHA domain, a central RING finger domain, and a C-terminal cysteine-rich domain (Fig. 2). The function of the FHA domain is largely unknown but is required for the checkpoint function and might be involved in the binding to phosphorylated proteins [19]. The RING finger domain is important for the ubiquitinating activity of CHFR and is able to form lysine 48- and lysine 63-linked polyubiquitination chains [25]. The cysteine-rich domain is important for the interaction between CHFR and target proteins [19–22]. Inside the cysteine-rich region, the RAR-binding zinc-finger (PBZ) is situated which is able to bind poly(ADP-ribose)PARP family members such as PARP-1.

Recently, it was shown that CHFR also ubiquitinates and regulates the expression of TOPK [33]. TOPK is a promitotic serine/threonine kinase that phosphorylates and inactivates downstream substrate PTEN, which will lead to activation of
AKT. By inhibiting TOPK, CHFR prevents the TOPK-induced activation of AKT and thereby blocks G2/M progression.

CHFR can also influence the mitotic checkpoint by functioning as an ubiquitin ligase that targets proteins not for degradation but for activation of signal transduction. By catalyzing the formation of noncanonical Lys63-linked polyubiquitin chains [25], CHFR was shown to activate the p38 stress kinase pathway, which will reverse chromosome condensation and induce a mitotic arrest [34]. Furthermore, CHFR indirectly inhibits the nuclear accumulation of cyclin B [24], thereby preventing the formation of the cyclin B1/cdk complex by the same mechanism, and also interacts with mitotic arrest deficient 2 (MAD2). MAD2 is a key protein in the spindle assembly checkpoint by its ability to sense improper spindle attachment and inhibit the anaphase-promoting-complex [35]. MAD2 is dependent upon binding to CHFR for its activation and transport to the kinetochore. In the absence of CHFR, MAD2 is not able to inhibit anaphase progression, which will result in mitotic defects [36].

Above-summarized data clearly show that CHFR is an important regulator of cell cycle progression. Since inactivation of CHFR promotes chromosomal defects and via activation of HDAC1 tissue invasion [28], CHFR malfunction is thought to play an important role in cancer progression and metastasis.

3 CHFR inactivation in cancer and its role as tumor suppressor

CHFR is more frequently inactivated in cancer than all other mitotic checkpoint control genes together [11]. Scolnick et al. were the first to report lack of CHFR expression in neuroblastoma and colorectal cancer cell lines [11]. Absence of CHFR in these cell lines resulted in a high mitotic index when exposed to microtubule stress compared to wild-type cancer cells, which was restored by reintroduction of functional CHFR. In a breast cancer cell line model, decreased CHFR expression resulted in an accelerated growth rate, enhanced invasiveness and amplified colony formation.

In order to study the physiological role of CHFR and its function in tumorigenesis, Chfr knockout mice have been generated [20]. Chfr knockout mice develop invasive lymphomas and solid tumors (lung, liver, gastrointestinal) after 40 weeks and have an increased susceptibility to chemical carcinogenesis [20]. Embryonic fibroblasts from Chfr-deficient mice show substantial aneuploidy and polyploidy. Therefore, CHFR inactivation is expected to participate in the acquisition of chromosomal defects and a chromosomal instability phenotype in cancer. In primary colorectal and breast cancer tissue, however, CHFR inactivation is not associated with chromosomal instability [37]. In colorectal cancer (CRC) and gastric cancer, CHFR inactivation is associated, however, with microsatellite instability (MSI) and MLH1

Fig. 2 CHFR gene and protein. Schematic representation of promoter CpG island methylation, mutation and polymorphisms with functional significance. of a CHFR gene encompassing 18 exons. CpG island is enlarged with CpG dinucleotides as vertical lines. TSS: transcription start site * mutation. # polymorphism. b CHFR protein consisting of 664 aminoacids. FHA: forhead-associated domain. RING: ringfinger domain. CR: cysteine-rich domain. PBZ: RAR-binding zinc-finger domain. Mutations in black, polymorphism in red.
promoter CpG island methylation. The mechanism underlying the association between CHFR inactivation and MSI is unknown but might be due to an underlying DNA methylation defect that causes promoter CpG island methylation of both CHFR and MLH1. Murine studies, however, demonstrated that simultaneous loss of Chfr and Mlh1 synergistically increased predisposition to cancer development, which implicates a more functional interaction [38].

Furthermore, a recent study shows an additional role for CHFR in regulating expression of pro-inflammatory chemokine interleukin-8 (IL-8). CHFR is able to inhibit the NFκB signaling pathway and IL-8, which subsequently resulted in decreased angiogenesis and cell migration [39–41]. Inactivation of CHFR triggers NFκB signaling activity and thereby accelerates angiogenesis and a metastatic phenotype, which is associated with a poor prognosis.

### 4 Genetic and epigenetic mechanisms of CHFR transcriptional silencing

In the last decade, disrupted CHFR expression has been described in multiple cancer tissues (Table 1). Although

| Cancer                      | Aberration                | Method, region analyzed       | Percentage of methylation | Ref  |
|-----------------------------|---------------------------|-------------------------------|---------------------------|------|
| Breast cancer               | Reduced expression        | IHC                           | 36 % (51/142)             | [70] |
|                             | Methylation               | demethylation and northern blot | 8 % (2/24) (cell lines)   | [71] |
| Bladder cancer              | Methylation               | MLPA                          | 18.7 % (17/91)            | [72] |
| Colorectal cancer           | Methylation               | COBRA, MSP, +281 to +51 bp    | 40 % (25/63)              | [73] |
|                             |                           | COBRA                         | 37 % (11/30)              | [74] |
|                             |                           | MSP, −240 to −73 bp           | 53 % (27/51) (adenomas)   | [71] |
|                             |                           | MSP, −226 to −82 bp           | 41 % (29/71)              | [75] |
|                             |                           | qMSP, +221 to +325 bp         | 26% (25/98)               | [76] |
|                             |                           | MSP, −240 to −73 bp           | 24 % (217/888)            | [77] |
|                             |                           | MSP, −226 to −82 bp           | 31 % (19/61)              | [67] |
| Gastric cancer              | Methylation               | COBRA                         | 39 % (24/61)              | [56] |
|                             |                           | MSP, −9 to +98 bp             | 35 % (25/71)              | [78] |
|                             |                           | COBRA                         | 44 % (19/43)              | [79] |
|                             |                           | MSP, −163 to −8 bp            | 52 % (24/46)              | [57] |
| Nasopharyngeal cancer       | Methylation               | MSP, −220 to −14 bp           | 61 % (22/36)              | [80] |
|                             |                           | MSP, −220 to −14 bp           | 59 % (31/53)              | [81] |
| Non-small cell lung cancer  | Reduces expression        | IHC                           | 39 % (16/41)              | [63] |
|                             | Methylation               | MSP, −220 to −14 bp           | 19 % (7/37)               | [42] |
|                             |                           | MSP, −220 to −14 bp           | 10 % (2/20)               | [74] |
|                             |                           | MSP, −220 to −14 bp/IHC       | 15 % (3/20)/39 % (69/157) | [53] |
|                             |                           | MSP, −195 to −99 bp/mRNA      | 32.4 % (100/308) (serum)  | [47] |
|                             |                           | MSP, −220 to −14 bp           | 10 % (16/165)             | [52] |
|                             |                           | MSP, −220 to −14 bp           | 3.1 % (1/32)              | [63] |
|                             |                           | Mutation                      | 6 % 3/53                  | [44] |
| Esophageal cancer           | Methylation               | MSP, −163 to −8 bp            | 16.3 % (7/43)             | [46] |
|                             | Copy number loss          | MSP, −227 to −86 bp           | 24 % (9/38)               | [82] |
|                             |                           | qPCR                          | 31 % (18/58)              | [45] |
|                             |                           | qPCR                          | 59 % (16/27)              | [45] |
| Cervical cancer             | Methylation               | MSP, +168 to 318              | 12 % (2/14)               | [59] |
| Hepatocellular cancer       | Methylation               | MSP, −225 to −85 bp           | 35 % (22/62)              | [83] |
| Biliary tract carcinoma     | Methylation               | MSP, −9 to +98 bp             | 16 % (6/37)               | [84] |
| Oral squamous cell cancer   | Methylation               | MSP, −220 to −14 bp           | 31 % (4/13)               | [85] |
|                             |                           |                               | 34.7 % (17/49)            | [86] |
| Cutaneous T-cell lymphoma   | Methylation               | CpG island microarray         | 19 % (5/28)               | [87] |
| Head and neck cancer        | Methylation               | COBRA                         | 30 % (16/54)              | [73] |
|                             |                           | MS-MLPA                       | 25 % (7/28)               | [88] |
| Endometrial cancer          | Methylation               | MSP, +168 to 318 bp           | 12 % (6/50)               | [60] |

IHC, immunohistochemistry; (MS)-MLPA, (methylation-specific) multiplex ligation-dependent probe amplification; MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis
promoter CpG island methylation is the most frequently occurring alteration leading to CHFR inactivation, genetic alterations have been observed occasionally. Seolnick and Halazonetis were the first to describe a sequence variation in the cysteine-rich domain of CHFR in osteosarcoma cell line U2OS [11] (Fig. 2). The variation consisted of a G to A transition leading to substitution of valine 539 by methionine and was initially interpreted as a missense mutation with functional impairment but turned out to be a polymorphism [42]. The relevance of variant genotypes was further studied in a series of 462 colorectal cancer patients and 245 healthy controls [43]. This study showed that the A allele of the GA variant was associated with a reduced CRC risk ($P = 0.02$; OR, 0.496; 95% CI, 0.279–0.883). Thereby, it was shown that polymorphisms in the CHFR gene can be used as indicator for colorectal cancer susceptibility.

Additional studies to identify structural variations in the CHFR coding sequence led to the identification of three missense mutations in non-small cell lung cancer (NSCLC); all three were associated with a defective mitotic checkpoint [44]. Two mutations target the FHA and RING finger domain and the third is located in the cysteine-rich region (Fig. 2b). These mutations, however, were observed in only 3 out of 53 patients. Loss of the chromosomal region harboring CHFR, 12q24.33, occurs more frequently. In esophageal adenocarcinomas (EAC), CHFR DNA copy number loss appears to occur in 59% of patients. In esophageal cancers as well, among which CRC (24–53%) and gastric cancer (35–52%) (Table 1). In NSCLC, CHFR promoter CpG island methylation occurs in approximately 10–40% of NSCLCs characterized by wild-type EGFR and KRAS in absence of ALK gene rearrangement, which implicates that CHFR promoter CpG island methylation occurs in a specific NSCLC subgroup [47].

Multiple studies have shown that CHFR promoter CpG island methylation can be detected not only in the primary cancers but also in blood (NSCLC) [47], stool (CRC) [48] and peritoneal fluid (gastric cancer) [49, 50]. This lends support to CHFR having promise as a diagnostic marker.

5 CHFR promoter methylation is associated with a poor prognosis and increased sensitivity to microtubule inhibitors

5.1 CHFR promoter CpG island methylation as prognostic marker

In recent years, it has become clear that CHFR promoter CpG island methylation is associated with a poor prognosis in multiple cancer types. In NSCLC, CHFR promoter CpG island methylation is associated with an increased risk of disease recurrence and poor survival [51–53]. In a series of 165 NSCLCs in which the CHFR promoter CpG island was methylated in 10% of patients and KRAS and EGFR mutation were found in 8% and 29% of cases, CHFR promoter CpG island methylation was the only molecular alteration that was associated with a shorter survival (log-rank test, $P = 0.0017$) [52]. In colorectal cancer, an association between CHFR promoter CpG island methylation and poor prognosis has been reported in two independent studies. Tanake et al. [54] showed in a retrospective study of 82 resected high-risk stage II or III CRC that CHFR promoter CpG island methylation (assessed by pyrosequencing) was associated with a shorter recurrence free survival (log-rank test, $P = 0.006$) and a reduced overall survival (log-rank test, $P = 0.07$). We also recently showed that CHFR promoter CpG island methylation is an indicator of poor survival in stage II BRAF wild type microsatellite stable CRC ($n = 66$, $P < 0.01$, HR = 3.89, 95% CI = 1.58–9.60) and validated these results in an independent prospective cohort study ($n = 136$, $P = 0.07$, HR = 2.11, 95% CI = 0.95–4.59) (Cleven et al., submitted).

Together, these studies indicate that CHFR promoter CpG island methylation is an indicator of an aggressive phenotype characterized by a high risk of disease recurrence and a shorter overall survival. Testing for CHFR promoter CpG island methylation may help to select patients with a poor prognosis. Future studies are needed to investigate which treatment or screenings approaches will improve survival for patients with CHFR inactivated cancers.

5.2 CHFR promoter CpG island methylation as predictor of taxane sensitivity

Although CHFR promoter CpG island methylation is associated with a poor prognosis, CHFR inactivation predisposes to an increased sensitivity to microtubule inhibitors (Table 2). Microtubule inhibitors such as docetaxel and paclitaxel disrupt normal microtubule dynamics during cell division by binding to the beta-tubulin subunits. This will lead to a failure of microtubule separation and apoptosis. As CHFR is able to block entry into prophase until chromosomal alignment is restored, CHFR inhibits the effect of taxanes. Accordingly, cells expressing CHFR are more
showed progressive disease. These results could, however, not be confirmed in a larger study of 41 gastric cancers in which promoter methylation status of CHFR was associated with a better clinical response compared to cancers with unmethylated CHFR. Treatment with 5-Aza-2′-deoxycytidine recovered CHFR expression and decreased the sensitivity to these agents immediately, an effect that was not observed for treatment with 5-fluorouracil, etoposide, cisplatin and doxorubicin. The same accounts for CHFR promoter CpG island methylation and treatment with paclitaxel in endometrial cancer cell lines and in NSCLCs.

Other preclinical evidence for CHFR as marker of taxane sensitivity comes from a recent retrospective study that analyzed CHFR inactivation and response to paclitaxel in metastatic NSCLC. CHFR promoter CpG island methylation was assessed with MSP and validated by methylation microarray and nuclear expression of CHFR was analyzed by immunohistochemistry. Although CHFR promoter CpG island methylation was observed in only 1/32 (3.1%) patients, 16/41 (37%) patients showed reduced nuclear staining of CHFR, indicating the presence of a CHFR repressive event other than promoter CpG island methylation that still needs to be elucidated. In this study diminished nuclear CHFR expression was associated with a better response to therapy (19% versus 52% progression at first restaging, \( P=0.033 \)) and a prolonged overall survival (9.1 versus 5.1 months, \( HR=0.28, 95\% CI=0.14–0.56 \)) compared to patients with high CHFR nuclear expression.

Finally, in colorectal cancer, a recently reported preclinical study showed increased sensitivity to taxanes in colorectal cancer cell lines both in vitro and in vivo. The correlation between CHFR expression and resistance to docetaxel was statistically significant (\( P=0.033 \)), with a 20-fold increase in median IC50 for cell lines that had measurable CHFR expression versus silenced cell lines.

Although randomized prospective clinical trials are needed before implementation into clinical practice, these studies together strongly support the evidence for CHFR inactivation as marker of taxane sensitivity.

### Table 2 CHFR inactivation as prognostic and predictive marker

| Cancer prognostic marker | Method | Ref |
|--------------------------|--------|-----|
| Lung cancer              | Reduced CHFR expression associated with poor prognosis (\( n=157 \)) | IHC | [53] |
|                          | CHFR promoter methylation associated with poor prognosis (\( n=208 \)) | MSP | [51] |
| Colorectal cancer        | CHFR promoter methylation associated with poor prognosis in stage II MSS BRAF wt CRC (\( n=66 \)). Confirmed in second independent series (\( n=136 \)) | MSP | [54] |
|                          | CHFR promoter methylation associated with reduced recurrence-free and overall survival (\( n=82 \)) | PS  |     |

| Predictive marker        | Method                      | Ref |
|--------------------------|-----------------------------|-----|
| Gastric cancer           | CHFR promoter methylation associated with increased sensitivity to paclitaxel (cell lines, \( n=4 \)) | MSP | [56] |
|                          | CHFR promoter methylation associated with increased sensitivity to paclitaxel (\( n=12 \)) | MSP | [57] |
|                          | CHFR promoter methylation and sensitivity to docetaxel or paclitaxel (\( n=41 \)) | COBRA | [58] |
| Cervical cancer          | CHFR promoter methylation associated with increased sensitivity to paclitaxel (cell lines, \( n=6 \)) | MSP | [59] |
| Oral squamous cell carcinomas | Silencing of CHFR with siRNA increases taxane sensitivity (cell lines) | siRNA | [85] |
| Lung cancer              | CHFR promoter methylation associated with increased sensitivity to paclitaxel (cell lines, \( n=69 \) and \( n=41 \)) | MSP | [62, 63] |
|                          | Unmethylated CHFR promoter associated with good response to EGFR TKIs (\( n=179 \)) | MSP | [47] |
|                          | Reduced CHFR expression predicts outcome to paclitaxel based therapy (\( n=41 \)) | IHC | [63] |
| Endometrial cancer       | CHFR promoter methylation associated with increased sensitivity to paclitaxel (cell lines, \( n=6 \)) | MSP | [60, 61] |

IHC, immunohistochemistry; MSP, methylation-specific PCR; PS, pyrosequencing; siRNA short interference RNA; TKI, tyrosine kinase inhibitors.
6 Conclusion

In the last decade, a substantial number of studies have been performed to investigate CHFR inactivation, usually due to promoter CpG island methylation, as biomarker to predict prognosis and response to microtubule inhibitors in a diversity of cancers. There is compelling evidence that reduced CHFR expression is a promising biomarker that can improve the management of multiple tumor types.

The clinical impact of CHFR promoter CpG island methylation as prognostic marker will be in the selection of patients with an aggressive phenotype. In stage II CRC, CHFR promoter CpG island methylation can help to identify patients with a worse prognosis that might benefit from adjuvant therapy. The same accounts for CHFR inactivation as predictive marker of taxane sensitivity where CHFR inactivation can help to select patients for taxane treatment. Furthermore, these results can be a rationale for studying the effect of taxane treatment in cancers with CHFR inactivation. A prospective trial to test this hypothesis in CRC is presently ongoing.

The clinical value of any biomarker, however, depends on the accuracy of the test. The majority of candidate biomarkers reported in literature do not reach clinical use mostly because they fail to pass the validation phase. This can be explained by intra- and inter-tumor heterogeneity, a technical inability to consistently verify the presence of the biomarker in patient’s material, and the lack of specificity for a particular disease. In most studies, CHFR promoter CpG island methylation is assessed by methylation-specific PCR (MSP). MSP is a very sensitive qualitative method that is able to detect aberrant methylation in minute amounts of DNA [65]. One important aspect of the technique is the region selected to be analyzed since not all regions within the CpG island have biological and clinical relevance [66]. In the literature, however, different locations within the promoter CpG island of CHFR have been analyzed (Table 1), which makes it difficult to compare results. CpG methylation within region −240 to −73 bp relative to the transcription start site of CHFR has shown to result in gene silencing [46] and is therefore proposed as core region of predictor methylation [67].

One of the technical challenges of MSP is a false positive result due to inadequacy of bisulfite treatment and mispriming especially when nested PCR or high numbers of PCR cycles are used [65]. Several alternative methods are available among which pyrosequencing [68]. Pyrosequencing permits a quantitative methylation analysis with single nucleotide resolution of the amplified region but also relies on amplification of bisulfite-converted DNA. One of the challenges of pyrosequencing, however, is that the technique requires a numeric cutoff value to define a positive methylation status, which is difficult in a clinical setting that is dependent upon biopsy specimens that are usually small and do not allow correction for tumor heterogeneity or involvement of normal tissues. The same accounts for other quantitative (q)MSP techniques.

Besides MSP, pyrosequencing and qMSP, recently also ultra-deep next-generation-based bisulfite sequencing has become available. Future studies are needed to evaluate the performance of the different techniques in assessing CHFR methylation status in a clinical setting. Since all associations between CHFR promoter methylation and clinical variables (Table 2) until now are consistently found with MSP, at present MSP is the more promising method of choice.

Furthermore, large, independent cohort studies and clinical trials are needed to validate the prognostic and predictive value of CHFR inactivation. These trials will need to be compared to established clinical markers such as Tumor-Node-Metastasis (TNM) classification system. Simon et al. have proposed a less time consuming design in which archival material of prospective trials is used to investigate the performance of a single biomarker [69].

In conclusion, the combination of the crucial role of CHFR in mitotic checkpoint control and a clear prognostic and predictive power highlights the clinical potential of CHFR as biomarker. Although work is still in progress, currently available results all point into the same direction and make CHFR inactivation, mostly due to promoter CpG island methylation, a biomarker with great potential and the development of clinical trials to validate its predictive and prognostic value, a priority.

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Conflict of interest

JH is consultant for MDx Health and receives research funding from MDx Health.

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