Crosstalk of methylation and tamoxifen in breast cancer (Review)

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Abstract. Tamoxifen is a widely used anti-estrogen drug in the endocrine therapy of breast cancer (BC). It blocks estrogen signaling by competitively binding to estrogen receptor α (ERα), thereby inhibiting the growth of BC cells. However, with the long-term application of tamoxifen, a subset of patients with BC have shown resistance to tamoxifen, which leads to low overall survival and progression-free survival. The molecular mechanism of resistance is mainly due to downregulation of ERα expression and abnormal activation of the PI3K/AKT/mTOR signaling pathway. Moreover, the downregulation of targeted gene expression mediated by DNA methylation is an important regulatory mode to control protein expression. In the present review, methylation and tamoxifen are briefly introduced, followed by a focus on the effect of methylation on tamoxifen resistance and sensitivity. Finally, the clinical application of methylation for tamoxifen is described, including its use as a prognostic indicator. Finally, it is hypothesized that when methylation is used in combination with tamoxifen, it could recover the resistance of tamoxifen.

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1. Introduction

As the most common female malignant tumor in the world, breast cancer (BC) has become a major health problem for women due to its high mortality and morbidity rates, and 5-year survival rate of <30% (1). The treatment of BC includes surgery, radiotherapy, chemotherapy, endocrine therapy and targeted therapy (1,2). According to different conditions and needs, it is necessary to develop personalized treatment plans to achieve the best treatment effect (3). Surgery is the main treatment for BC, including radical mastectomy breast-conserving surgery and breast reconstruction (1). Surgical intervention can effectively remove the tumor and reduce the risk of recurrence (4). After surgery, adjuvant therapy (such as radiotherapy and chemotherapy) may be required according to tumor stage, tumor grade, molecular typing and patient status (3). Radiation therapy refers to the use of high-energy rays to kill cancer cells and is often used as an adjunct treatment after surgery to reduce the risk of recurrence (4). For several patients who are inoperable or at high risk of surgery, radiation therapy can also be used as the primary treatment (5). Chemotherapy is a method of killing cancer cells with drugs and can be used to shrink tumors before surgery (6). Endocrine therapy is mainly aimed at patients with hormone receptor-positive BC, which inhibits hormones to block cancer cell growth (3,7). Endocrine therapy drugs include anti-estrogen drugs and aromatase inhibitors (3,7). Targeted therapies are treatments that target specific cancer cells, such as trastuzumab for HER2-positive BC (2), and compared with traditional therapy, has higher pertinence and lower side effects (2).

Endocrine therapy for BC is a long-term treatment (7). Postoperative adjuvant therapy for patients with early BC generally occurs over 5 years, but in some cases may extend to 10 years (8). Risk stratification is clinically performed for early patients with hormone receptor-positive BC. Low-risk patients only need to be treated with a single drug for 5 years, while high-risk patients need intensive treatment (8). On the one hand, endocrine therapy for high-risk patients is supplemented by intensive treatment with ovarian function inhibition or CDK4/6 inhibitors (3). On the other hand, the duration of treatment for high-risk patients was extended to 10 years (7). For medium-risk patients, it is necessary to further determine whether endocrine therapy should last for 5 or 10 years and whether a combination regimen or a single drug regimen is selected according to the clinical characteristics of patients (9).

Given that the sources of hormones in premenopausal and postmenopausal women are different, the choice of therapeutic drugs needs to be tailored to different populations (9). For patients with BC with a relatively low risk of recurrence after surgery, selective estrogen receptor modulator (SERM) therapy is often given, of which tamoxifen is a commonly used drug (10).

Tamoxifen is a synthetic non-steroidal anti-estrogen drug, widely used in patients with ER-positive BC, which prolongs the survival of patients (11). Although tamoxifen has made important clinical advances in endocrine therapy, primary and acquired drug resistance has limited its clinical efficacy (12). Downregulation of ERα expression, up-regulation of ERβ expression, the activation of signaling pathways (such as the PI3K/AKT/mTOR signaling pathway), and the activation of certain key proteins and RNA can all lead to tamoxifen resistance in patients with BC (12-14).

Methylation is a common chemical modification in organisms, which affects the expression of DNA, RNA and proteins (15). Enzymes that catalyze DNA methylation are termed DNA methyltransferases (DNMTs) (16). DNMTs are highly expressed in tamoxifen-resistant patients and are important factors for tamoxifen resistance in BC (17). In addition, Jahangiri *et al* (18) found that promoters of DNMTs are demethylated, which leads to overexpression of DNMTs, promoting tamoxifen resistance and the recurrence of BC. Therefore, the promoters of tamoxifen-resistant cell lines have higher methylation levels, resulting in reduced expression of genes including nuclear receptor-interacting protein 1, human homolog of the Drosophila headcase and the mitochondrial fission protein 1 (19).

In the present review, methylation is introduced in detail, including DNA methylation, RNA methylation and protein methylation. After which, tamoxifen is described and divided into its clinical use and tamoxifen resistance-related mechanisms. In addition, the effect of methylation on tamoxifen resistance, and clustering on the ER and PI3K/AKT/mTOR signaling pathways is described. Furthermore, tamoxifen-induced methylation is also elucidated. Finally, the clinical applications of methylation are also described, with a focus on prognostic analysis and potential clinical drug discovery.

2. Methylation

Epigenetic modifications mainly include chemical modifications that occur on DNA, RNA and proteins (20). In general, chemical modifications that occur on RNA are termed post-transcriptional modifications and those that occur on proteins are termed post-translational modifications (21). These epigenetic modifications do not change the genetic code of genes, but they have an important effect on gene expression (21). DNA modification is broadly divided into DNA methylation, which silences the expression of genes, and DNA phosphorylation, which affects the structure and function of DNA (22). RNA modifications include N6-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am) and N1-methyladenosine (23). Among them, m6A, the methylation of the N-6 adenosine base, is the most widely studied, affecting splicing, output, stability, degradation and translation, and thus affecting gene expression (24). As for protein modifications, phosphorylation is one of the most abundant post-translational modifications, which is involved in the regulation of cell signal transduction, protein-protein interaction and gene transcription (25). Ubiquitination, acetylation and methylation can also affect protein-protein interactions, protein stability, subcellular localization or enzymatic activity (26).

Methylation refers to the process of transferring methyl groups from active methyl compounds to other compounds, which results in the formation of various methyl compounds, or chemical modifications in proteins or nucleic acids to form methylated products (27,28). Based on the substrate to which the methyl group binds, methylation is mainly divided into DNA methylation, RNA methylation and protein methylation (Fig. 1) (28). Methyl donors are mainly derived from one-carbon metabolism (29), which is a one-carbon unit metabolic process, including folate cycle, the methionine cycle and trans-vulcanization pathway, wherein the methionine cycle is the main pathway to produce methyl donors (29). In the methionine cycle, one carbon unit can be used to remethylate homocysteine to produce methionine (30). Methionine produces s-adenosylmethionine (SAM) with the help of methionine adenylyl transferase, and transfers methyl groups to biomolecules, including proteins, nucleic acids and lipids (30). The change of methylation status is caused by the difference in the activity of methyltransferase and demethylase (31), with SAM as the main methyl donor of these enzymes (31). Alterations in the methylation of proteins, nucleotides and metabolites can lead to the occurrence of cancer (31).

DNA methylation. DNA methylation is a chemical modification process, in which the cytosine of vertebrate CpG dinucleotides is catalyzed by DNMTs and acquires a methyl group using SAM as the methyl donor (27). DNA methylation occurs on the N-6 of adenine, N-7 of guanine, and C-5 of cytosine (31). However, DNA methylation primarily occurs on the cytosine of 5'-CpG-3', resulting in the formation of 5-methylcytosine (27). A portion of CpG dinucleotides is dispersed throughout the genome, while another portion occurs in dense clusters of CpG islands (32). In normal tissues, most of the dispersed CpG is methylated, while CpG islands tend to be unmethylated (32). Normally, CpG dinucleotides in relatively useless or unfavorable genomes are rare and are always in a methylated state (33). By contrast, CpG islands rich in CpG dinucleotides with a size of 100-1,000 bp in the genome are always unmethylated, and CpG islands are often located near the transcriptional regulatory region and are associated with more than half of the genes encoded (33). Therefore, it is necessary to study the methylation of CpG islands in the transcription region.

The hypermethylation of CpG sites in enhancers or promoters leads to transcriptional silencing, whereas the hypomethylation of CpG sites usually leads to transcriptional activation, so methylation regulates gene expression through gene transcription (27). DNA methylation is mainly dependent on the DNMT family, which has five members including DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (27). DNMT1 is primarily involved in DNA methylation, which is required to silence tumor suppressor genes (16). DNMT2 is an RNA methyltransferase that modifies cytosine residues in certain tRNA anticodon rings (16). The key role of DNMT3A

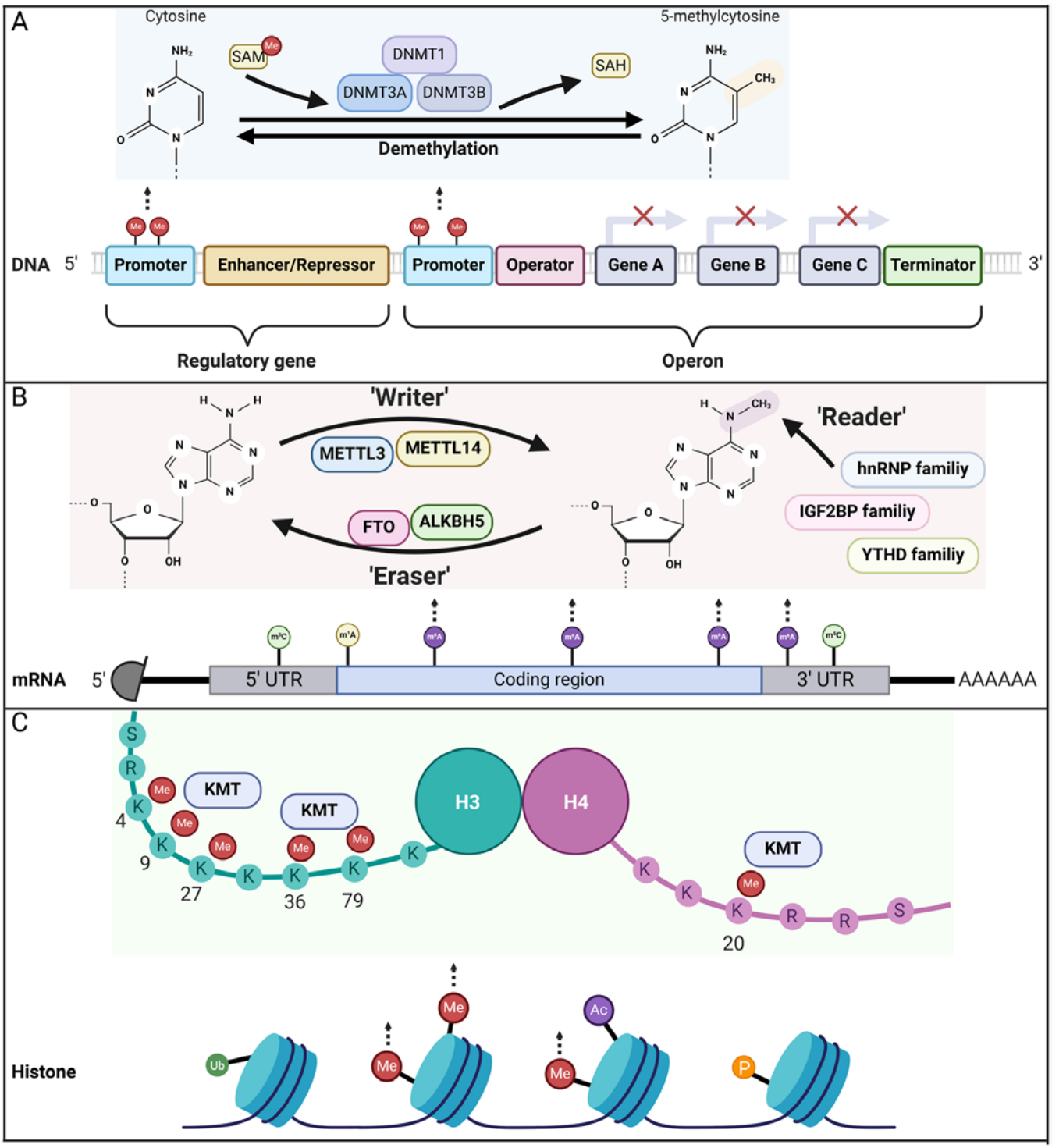

Figure 1. Methylation includes DNA methylation, RNA methylation and protein methylation. (A) DNA methylation occurs mainly on the promoter of DNA, resulting in inhibition of DNA expression. (B) The m6A methylation is the most common form of RNA methylation that stabilizes mRNA. (C) Histone methylation mainly occurs in H3 and H4. SAM, s-adenosylmethionine; SAH, S-adenosylhomocysteine; DNMT, DNA methyltransferase; METTL, methyltransferase-like; FTO, obesity-associated protein; ALKBH5, AlkB homolog 5; KMT, lysine-specific methyltransferase.

and DNMT3B is *de novo* methylation (34) Although DNMT3L is a member of the DNMT3 family, it does not have methyltransferase activity (34), however, DNMT3L can assist DNMT3A/B-mediated *de novo* methylation (35). In addition, DNMT3A includes two isoforms, while DNMT3B contains >30 isoforms, all of which have methylation activity and conserved C-terminal domains (35). In conclusion, DNMT1 participates in DNA methylation, whereas DNMT3A and DNMT3B, whose primary role is *de novo* methylation, could also be involved in DNA methylation (16).

DNA methylation has been detected at a very early stage, and it is also a common epigenetic phenomenon, which plays an important role in the maintenance of the stability of the genome and the regulation of normal physiological functions (36). In

the process of tumorigenesis, there is a decrease in the DNA methylation level of most genes and an increase in the methylation level of CpG islands of some genes, including DNA repair genes, cell cycle genes and apoptosis genes (36). Low levels of DNA methylation not only lead to reduced genomic stability and mutation rates, but also abnormally activate the expression of multiple oncogenes, such as chromatin modifiers and transcription factors (37). By contrast, high levels of DNA methylation indirectly induce malignant tumors by decreasing the transcriptional activity of tumor suppressor genes and then affecting the expression (37).

RNA methylation. In addition to DNA methylation, RNA can also be methylated to participate in the regulation of gene expression (38). RNA methylation is a post-transcriptional modification that transfers methyl groups from methyl donors to RNA bases with the help of RNA methyltransferase, thereby regulating stability, splicing, localization and translation (38,39). RNA methylation modification is reversible and occurs in different types of RNA, including microRNA (miRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA) and small nucleolar RNA (38,40).

RNA methylation occurs at the m6A methylation modification at adenylate N6, which is the most abundant RNA modification found in eukaryotes (41). The m6A methylation has its methylase (writer), demethylase (eraser) and methylation recognition protein (reader), which synergistically mediate RNA methylation (41). The m6A methylation in mRNA is mainly catalyzed by the METTL3/METTL14 complex (40). METTL3 primarily acts as a catalyst, while METTL14 is an allosteric activator that helps bind to the target RNA (40). METTL3 is mainly located in the nucleus, but METTL3 is present in the cytoplasm in several cells (42). The majority of m6A methylation occurs in rRNA, mediated by the N6-adenosine-methyltransferase zinc finger CCHC-type containing 4 and methyltransferase-like 5/tRNA methyltransferase activator subunit 11-2 complexes (41). In addition, m6A methylation of snRNA U6 is catalyzed by METTL16, which also catalyzes small amounts of mRNA and other non-coding RNA, in particular microRNAs and lncRNAs (43). The m6A demethylase is an enzyme that converts m6A to adenylate, thereby removing m6A, and this process is catalyzed by obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5) (43). FTO catalyzes the demethylation of 3-methyluridine, m6A and m6Am, but which RNA is mainly targeted is still controversial (44). Furthermore, m6Am methylation in snRNA may be a specific target of FTO, and its mechanism remains to be further revealed (44). Although ALKBH5 is a demethylase, it has no effects on m6Am (45). As for m6A recognition proteins, it is usually recruited by m6A methylation, which affects mRNA functions such as localization (46). The m6A methylation recognition proteins include the YTH domain family (YTHDF1/2/3 and YTHDC1/2), hnRNP family (hnRNPC, hnRNPG and hnRNPA2B1) and insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs) (46). The YTH domain can directly bind to m6A, and YTHDF2 can recruit complexes to promote the degradation of m6A-modified mRNA (47). By contrast, IGF2BPs can enhance the stability and translation efficiency of m6A-modified mRNA (48). HnRNPC selectively recognizes m6A-induced splicing in the secondary structure of mRNA (49).

Protein methylation. Protein methylation refers to the methylation of arginine or lysine in the protein (50). Arginine can be methylated once or twice by arginine methyltransferase, whereas lysine can be methylated 1-3 times by lysine methyltransferase (51). Among them, arginine methyltransferase transfers two methyl groups to the same nitrogen atom of the arginine polypeptide to form asymmetric dimethylarginine, and one methyl group is added to each nitrogen terminal to form symmetric dimethylarginine (51).

Among the protein methylation, the methylation of histones has been studied most extensively (51). Histone methylation is the methylation of lysine or arginine residues on the N terminal of the H3 or H4 histones (51,52). The effects of histone methylation are mainly reflected in heterochromatin formation, gene imprinting, X chromosome inactivation and transcriptional regulation (53). This process is catalyzed by histone methyltransferase (HMT), which can be divided into histone lysine methyltransferase and protein arginine methyltransferase (PRMT) (53). H3 lysine (H3K) 4, 9, 27, 36, 79 and H4 lysine (H4K) 20 can be methylated, with histones H3K4 and H3K9 being the two most common modification sites (54). Likewise, the histone lysine residues can undergo single, double or trimethylation, while the arginine residues undergo only mono-methylation and di-methylation (54). The methylation of different sites of histone H3 and H4 and the amount of methylation have different influences on transcriptional regulation. In most cases, H3K9me3, H3K27me3 and H4K20me2/3 mediate transcriptional suppression, while H3K4me1/3, H3K9me1, H3K27me1, H3K36me1/3 and H3K79me1/3 mediate transcriptional activation (54).

In addition to HMT, there are also histone demethylases. Histone demethylases can be divided into two families: i) The lysine-specific demethylase (LSD) family; and ii) the Jumonji C domain-containing (JMJD) family (55). LSD can specifically remove the mono-methylation and di-methylation of histones H3K4 and H3K9, while JMJD can remove the tri-methylation of lysine (55). Therefore, methylase and demethylase together promote the stability and dynamics of histone methylation.

3. Tamoxifen

Tamoxifen in a clinical setting. In 1978, the FDA approved tamoxifen for the treatment of patients with advanced BC (56). The 2010 ASCO guidelines recommend tamoxifen as the standard for premenopausal women, and at present has become an important drug for adjuvant endocrine therapy of BC (57).

Currently, tamoxifen is mainly used in patients with hormone receptor-positive BC, and it is mostly used in premenopausal patients or postmenopausal patients who cannot tolerate aromatase inhibitors (58). In addition, tamoxifen can also be used in the following situations: i) To treat recurrent metastatic BC and ovarian cancer (59); ii) for adjuvant treatment of lymph node-negative BC after breast surgery, and radiotherapy and chemotherapy (60); iii) it is used for the adjuvant treatment of postmenopausal breast surgery and lymph node-positive BC after radiotherapy and chemotherapy (60); and iv) for patients with ductal carcinoma *in situ* after breast surgery and radiation as it may reduce the risk of invasive BC (61). Moreover, clinical studies suggest that for women with a family history of BC, the use of tamoxifen can reduce their risk of BC by

more than one-third (62-65). Therefore, tamoxifen is also used for BC prevention. The conventional dose of tamoxifen for BC is 20 mg, but it can also be increased to a maximum of 40 mg/day (66), and can be taken by mouth as a single dose or in two equal doses (66). The conventional endocrine treatment cycle is 5 years, and the dose of the drug and the treatment drug can also be changed after 2 years of tamoxifen treatment (67). However, long-term use of tamoxifen leads to a series of adverse reactions, including secondary estrogen effects, gastrointestinal reactions, neuropsychiatric symptoms and bone marrow suppression (68). Secondary antiestrogenic effects include facial flushing, vulvar pruritus, menstrual disorders, amenorrhea, increased leucorrhea and vaginal bleeding (68,69). Gastrointestinal reactions include loss of appetite, nausea, vomiting and diarrhea (69). Neuropsychiatric symptoms include headache, dizziness and depression (68). Furthermore, several patients may suffer from transient leukopenia and thrombocytopenia (69). In addition, large doses and long-term application can lead to visual impairment, rash, hair loss, weight gain and liver dysfunction (70). However, most patients experience relatively mild symptoms, which can be overcome and will be relieved after stopping the drug in later stages (70). However, it must be noted that the use of tamoxifen may be associated with more serious adverse effects including venous thrombosis and endometrial cancer (EC), but this is less likely to occur (71). Therefore, patients should have a gynecological examination once a year during the use of tamoxifen (71).

Tamoxifen is an estrogen receptor modulator, and its main target is ERα (72). ERα promotes intracellular signaling primarily through estrogen/ERα-mediated nucleus-initiated steroid signaling (genomic signaling) (72). The process can be divided into three steps. First, estrogen enters the cell through diffusion or *in situ* synthesis. Second, estrogens bind to ERα in the nucleus, which activates and forms ERα homodimers or heterodimers (73). Finally, the activated ERα binds to the DNA enhancer estrogen response element (ERE), so that the ERα-ERE complex promotes the formation of the transcription initiation complex and induces transcription (74). In addition to the ERE mechanism, ERα binds to other transcription factors and then binds to the activating protein 1 at the activating region of the target genes to regulate gene transcription (73). By binding with ERα in BC, tamoxifen blocks the binding of estrogen to ERα, making estrogen inactive, either blocking stimulation of transcription or weakening its effect, thus inhibiting the occurrence and development of BC (75). In addition, tamoxifen can up-regulate the production of transforming growth factor β, which makes tamoxifen suitable for patients with osteoporosis (76).

Tamoxifen resistance. After long-term use of tamoxifen, some patients will develop tamoxifen resistance, and its specific molecular mechanism is complex and diverse. It includes downregulation of ERα, up-regulation of ERβ, the emergence of BC stem cells (BCSCs) and activation of the signaling pathway (Fig. 2) (12).

A number of studies indicate that the inhibition of ERα expression may be the main cause of resistance to endocrine therapy (12,14). Given that the mechanism of tamoxifen is mainly mediated by ERα and the expression of ERα is a good predictor of response to tamoxifen, loss of expression of ERα has been widely recognized as a major factor in tamoxifen resistance (12). Loss of ERα expression may be associated with methylation of CpG islands and increased histone deacetylation, resulting in a more compact nucleosome structure that restricts ERα transcription and thus limits the efficacy of tamoxifen (77). Moreover, loss of ERα has been associated with tumor invasion and suggests a poor prognosis. In addition, a study showed that re-expression of ERα can reverse tamoxifen resistance in MCF-7 cells, suggesting that loss of ERα expression may be an important mechanism of tamoxifen resistance (12). Similarly, ERα protein point mutations (such as K303R) enhance ERα-mediated cell growth, which is caused by increased estrogen sensitivity and alters various cellular signaling pathways (78). Since these signaling pathways normally downregulate ERα signaling, the ERα signaling pathway is inhibited and patients become resistant to tamoxifen (78). There are also multiple ERα mutations in the ligand-binding region of ERα, and in the absence of ligands, these mutations promote ERα conformational changes that lead to the proliferation of hormone-independent tumor cells and resistance to tamoxifen (12). ERβ is a product of *ESR2* located at chromosome 14q.21 (79). ERβ and ERα have 96% homology in the DNA binding region and 59% homology in the ligand binding region (79). Interestingly, the activation of ERβ expression enhances tamoxifen resistance, which is mainly determined by the physiological function of ERβ (12). ERβ is highly homologous to ERα and binds to estrogen with a similar affinity to ERα (80). ERα and ERβ respond in different ways depending on the ligands and the responding elements (81). ERα up-regulates the expression of genes related to cell growth (81). However, ERβ is more abundant than ERα expression in normal breast cells (72). In ERβ knockout mice, the proliferation of mammary cells accelerated, whereas in ERα knockout mice, the mammary cells shrank (72). ERβ has various effects on ERα-related regulatory genes, which include regulating the expression of the *ERα* gene and enhancing or weakening the effect of ERα (82). Overall, ERβ inhibited ~70% of ERα-regulated genes (82). In contrast to tamoxifen-sensitive tumors, ERβ was ~2 times higher in tamoxifen-resistant tumors than ERα (12). Thus, ERβ expression activated by ERβ-selective agonists, such as LY500307, may promote tamoxifen resistance by inhibiting ERα (72). However, studies have also shown that some ERβ splicing variants can lead to the development of tamoxifen resistance, which is associated with a poorer prognosis in advanced BC (79,83-85). In addition, the expression of ERβ in tumor infiltrating leukocytes was much higher than that of ERα in the tumor microenvironment, suggesting that ERβ may alter the tamoxifen response by influencing immune cells (72).

Except for ER, abnormal activation of signaling pathways can also lead to tamoxifen resistance. The related signaling pathways include the PI3K/AKT/mTOR signaling pathway, the NF-κB signaling pathway and the Hedgehog signaling pathway (75,78,86). The most important of these is the PI3K/AKT/mTOR signaling pathway (87). The PI3K/AKT/mTOR signaling pathway regulates multiple biological processes, including cell proliferation, apoptosis and metabolism (87). This signaling pathway is a complex regulatory network involving multiple components including

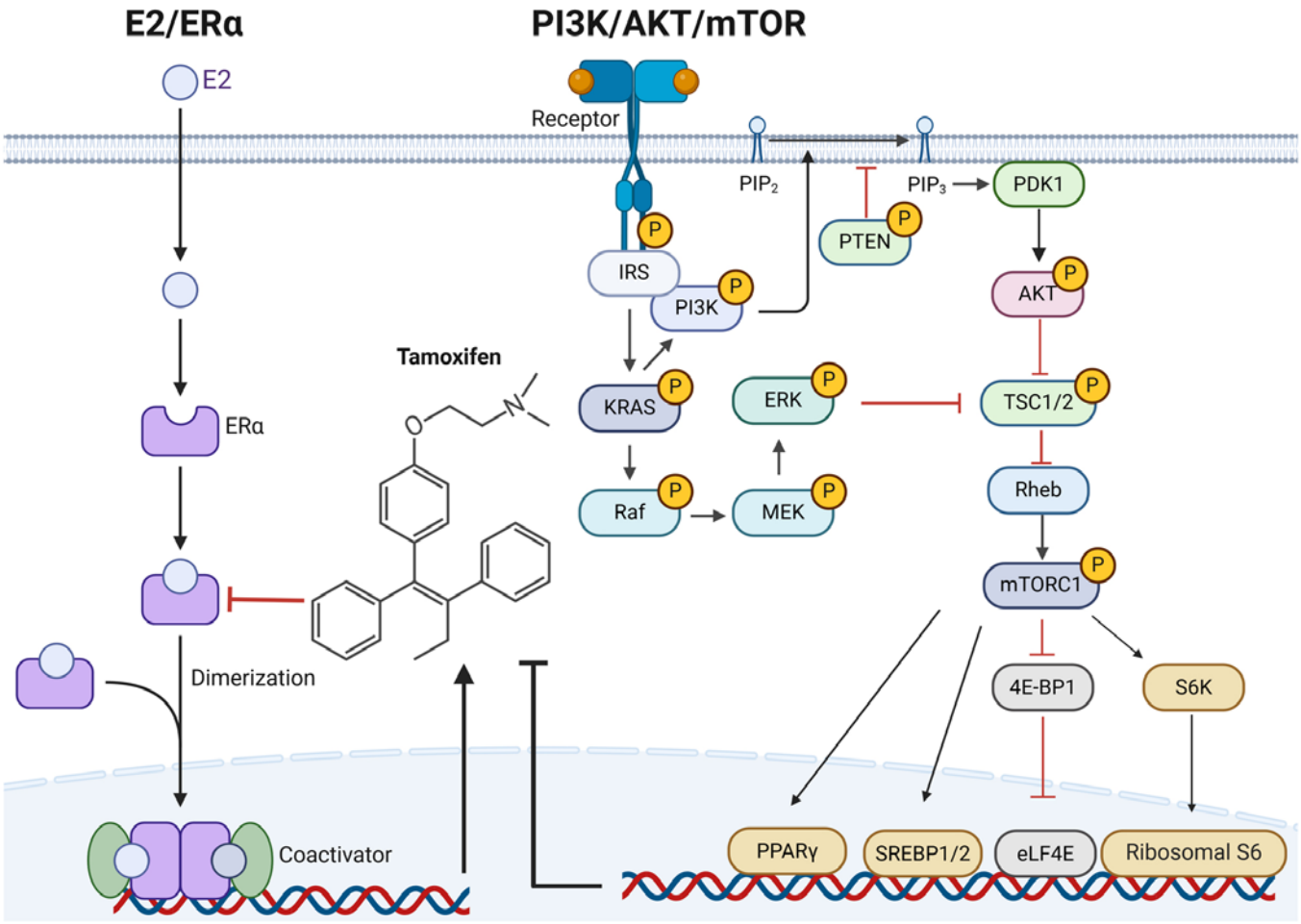

Figure 2. Mechanism of tamoxifen resistance. The activation of the E2/ERα signaling pathway contributes to tamoxifen sensitivity. By contrast, the activation of the PI3K/AKT/mTOR signaling pathway leads to tamoxifen resistance. E2, oestrogen; ERα, estrogen receptor α; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; IRS, insulin receptor substrate; PTEN, phosphatase and tensin homolog; PDK1, 3-phosphoinositide-dependent protein kinase 1; TSC, tuberous sclerosis complex; mTORC1, target of rapamycin complex 1; 4E-BP1, eukaryotic translation initiation factor 4E binding protein; S6K, p70/85 S6 kinase; PPARγ, peroxisome proliferator-activated receptor gamma; Elf4e, eukaryotic initiation factor 4E.

upstream regulation, internal regulation and downstream regulation. Its upstream regulation is receptor tyrosine kinase (RTK) (88). When the upstream protein is activated by external stimuli, such as growth factors, the RTK undergoes a conformational change that activates its tyrosine kinase activity (88). The activated RTK binds directly to p85, the subunit of PI3K, causing p85 to release its inhibition on p110, thereby activating PI3K (89). Activated PI3K converts phosphatidylinositol diphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3) (89). After which, PIP3 attracts PDK1 and AKT, enabling PDK1 to phosphorylate AKT at Thr308, thereby activating AKT (90,91). In addition to AKT, the inhibitory factor of internal regulation is PTEN (92). PTEN can dephosphorylate PIP3 to PIP2, which limits the activation of the signaling pathway (92). Therefore, the absence or abnormal function of PTEN leads to overactivation of the PI3K/AKT/mTOR signaling pathway (93). Activated AKT phosphorylates multiple downstream target proteins and thus participates in cellular physiological processes. Phosphorylated proteins targeted by AKT include mTOR, FOXO, GSK3, Bcl-2 and NF-κB (88,94-96). Activation of the PI3K/AKT/mTOR signaling pathway was found to cause tamoxifen-resistant cells to become resistant to DNA-damaged chemotherapy by up-regulating BARD1 and BRCA1, suggesting that the PI3K/AKT/mTOR signaling pathway is important in the treatment of BC (97). In addition, high expression of phosphorylated AKT is associated with poor prognosis, and the inhibition of AKT expression is conducive to activation of drug-resistant cells (87). While multiple drugs targeting the PI3K/AKT/mTOR signaling pathway have been used to overcome tamoxifen resistance, inhibiting this pathway will activate the compensatory mechanism due to the complexity of the PI3K/AKT/mTOR pathway, which limits the effects of inhibitors (87).

The cancer stem cell model is another important factor in BC resistance to tamoxifen (89). BCSCs refer to a small subset of BC cytoplasmic cells that have the ability to self-renew, differentiate and perform tumorigenesis (98). Clinically, BCSCs are considered to be relatively resistant to radiotherapy, chemotherapy and molecularly targeted therapy, leading to the development of drug resistance and cancer recurrence (99). $CD44^{+}/CD24^{-}$ and $ALDH^{+}$ are the most common molecular markers of BCSCs, and other surface proteins are also considered markers of BCSCs, such as CD133, CD61, C-X-C

Table I. Effect of *ESR1*-related methylation on tamoxifen response.

Gene[a]	Enzyme[b]	Type[c]	Number[d]	ERα[e]	Tamoxifen[f]	(Refs.)
ESR1	MLL3 and SET1A	H3K4 me3	me3	Up	Sensitivity	(116,122)
ESR1	DNMT1	DNA promoter	NA	Down	Resistance	(191,192)
ESR1	DNMT3B	DNA promoter	NA	Down	Resistance	(117)
ESR1	NA	DNA promoter	NA	Down	Resistance	(118)
ESR1	TET2	demethylation	NA	Up	Sensitivity	(207)
ERβ	NA	DNA promoter	NA	Down	Resistance	(120)
ESR1	MLL1	H3K4me3	me3	Up	Sensitivity	(121)
ESR1	NA	DNA enhancer	NA	Down	Resistance	(123)
ESR1	EZH2	H3K27 me3	me3	Down	Resistance	(124)
GREB1	DNMT1, DNMT3B	DNA promoter	NA	Down	Resistance	(125)
UCHL1	TET1, TET3	Demethylation	NA	Down	Resistance	(126)
p21	EZH2	H3K27me3	me3	Down	Resistance	(131)
WT1	NA	DNA promoter	NA	Down	Resistance	(132)
ID4	NA	DNA promoter	NA	Up	Sensitivity	(133)
miR-27b	NA	DNA promoter	NA	Up	Resistance	(134)
NAT1	NA	DNA promoter	NA	Down	Resistance	(138)
ELOVL2	NA	DNA promoter	NA	Down	Resistance	(140)
PRA	NA	DNA promoter	NA	Down	Resistance	(141)
PAX2	NA	DNA promoter	NA	Up	Resistance	(144)
E-cadherin	NA	DNA promoter	NA	Up	Resistance	(145)
MMP1	NA	DNA promoter	NA	Down	Sensitivity	(146)
PTPRO	NA	DNA promoter	NA	Up	Resistance	(149)

[a]Target genes that are methylated or demethylated; [b]enzymes that mediate methylation or demethylation of target genes; [c]types of methylation, which mainly includes methylation on DNA promoters and methylation on lysine of histones; [d]the number of times the histone is methylated or the number of methyl groups on the lysine; [e]effects of targeted gene methylation on the expression or the function of ERα protein; [f]effects of targeted gene methylation on the tamoxifen response. ESR1, estrogen receptor 1; GREB1, gene regulated by estrogen in breast cancer 1; UCHL1, ubiquitin C-terminal hydrolase L1; WT1, Wilms' tumor 1; ID4, inhibitor of differentiation 4; miR, microRNA; NAT1, N-acetyltransferase type 1; ELOVL2, elongation of very long chain fatty acids-like 2; PRA, progesterone receptor α; PAX2, paired box 2; MMP1, matrix metalloproteinase 1; PTPRO, protein tyrosine phosphatase receptor type O; MLL, mixed-lineage leukemia protein; DNMT, DNA methyltransferase; TET2, ten-eleven translocation methylcytosine dioxygenase 2; EZH2, enhancer of zester homolog 2; H3K4, histone H3 lysine 4; H3K27, histone H3 lysine 27; NA, not applicable.

chemokine receptor type 4 (CXCR4) and microsatellite instability (100,101). Ectopic expression of ERα mutations (such as Y537S, Y537N and D538G) enriches $CD44^+/CD24^-$ cells, increases the formation of mammospheres, and upregulates a variety of stemness genes such as octamer-binding protein-4, SRY-box transcription factor 2, SRY-box transcription factor 9 (SOX9) and B-cell-specific Moloney murine leukemia virus integration site 1, thereby promoting BCSC enrichment and leading to endocrine resistance (102). Furthermore, PI3K/AKT/mTOR, Notch, Wnt and Hippo signaling pathways can also promote the enrichment of BCSCs, thus leading to the generation of endocrine therapy resistance (103). Based on the study of these BCSCs models, a number of therapeutic options are being gradually introduced to the clinic, including multi-drug chemotherapy and molecular targeted therapy (100). For example, the γ-secretase inhibitor MK-0752 in combination with tamoxifen or letrozole in patients with early-stage BC, and the γ-secretase inhibitor RO4929097 in combination with exemestane in patients with advanced BC are already in clinical trials (104-107).

In addition to the aforementioned three mechanisms, other proteins and RNAs play a crucial role in the complex network of tamoxifen. For example, SOX9, HDAC1, SIRT1 and HIF-1αcan promote BC resistance to tamoxifen (108-111). Furthermore, several miRNAs (such as miR-342 and miR-375) and LncRNA can also alter the BC response to tamoxifen through multiple mechanisms (112-114).

4. Effects of methylation on the tamoxifen response

Estrogen receptor. In the majority of cases, methylation affects tamoxifen sensitivity and resistance by altering transcription of the *ESR1* gene and ERα protein-mediated transcription (115-118). In general, up-regulation of ERα protein or enhanced ERα-mediated transcriptional regulation promotes tamoxifen sensitivity (Table I; Fig. 3) (12). By contrast, inhibition of ERα protein expression or reduction of ERα-mediated transcriptional regulation may promote tamoxifen resistance (Table I; Fig. 3) (12).

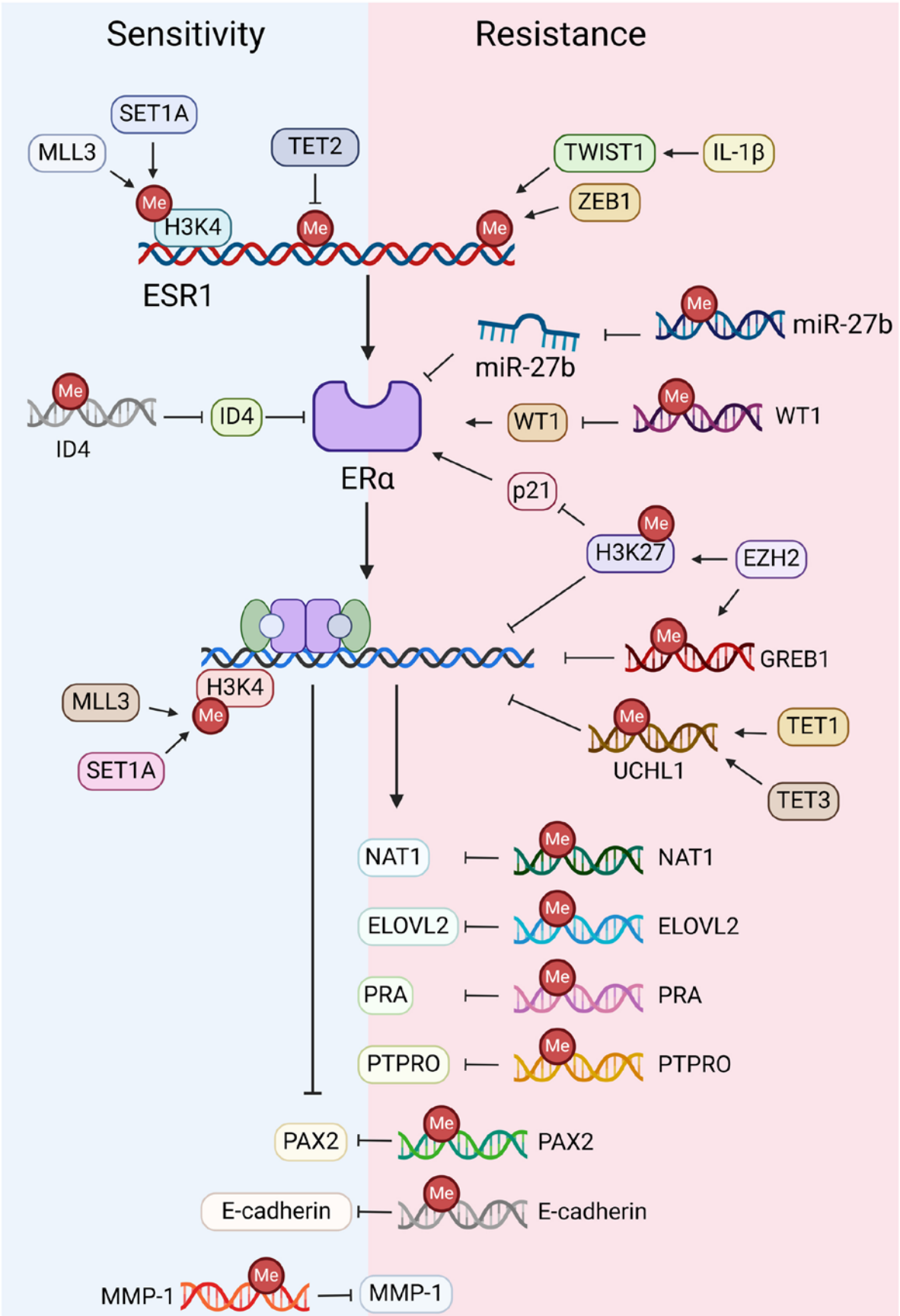

Figure 3. Effects of ERα methylation on tamoxifen. Methylation-induced downregulation of ERα expression or ERα signaling leads to tamoxifen resistance. MLL, mixed-lineage leukemia protein; H3K4, histone H3 lysine 4; TET2, ten-eleven translocation methylcytosine dioxygenase 2; ZEB1, zinc finger E-box-binding homeobox 1; IL-1β, interleukin-1β; ESR1, estrogen receptor 1; ID4, inhibitor of differentiation 4; miR, microRNA; WT1, Wilms' tumor 1; EZH2, enhancer of zester homolog 2; H3K27, histone H3 lysine 27; GREB1, gene regulated by estrogen in breast cancer 1; UCHL1, ubiquitin C-terminal hydrolase L1; NAT1, N-acetyltransferase type 1; ELOVL2, elongation of very long chain fatty acids-like 2; PRA, progesterone receptor α; PTPRO, protein tyrosine phosphatase receptor type O; PAX2, paired box 2; MMP1, matrix metalloproteinase 1.

Firstly, the methylation of the *ESR1* promoter affects *ESR1* transcription and ERα protein expression, thus playing an important role in BC response to tamoxifen. DNA methyltransferase-mediated hypermethylation of the *ESR1* promoter is associated with poor prognosis and indicates the development of tamoxifen resistance (115). The methylation of H3 is also involved in ESR1 transcription and thus affects the tamoxifen response. MLL3 and SET1A-mediated methylation of histone H3K4 enhance *ESR1* transcription, thereby promoting sensitivity to hormone therapy (116). In addition, ZEB1 and IL-1β also promote tamoxifen resistance by promoting the methylation of *ESR1* (117,118). ZEB1 induces hypermethylation of the *ESR1* promoter and silencing of ERα by forming the ZEB1/DNMT3B/HDAC1 complex on the *ESR1* promoter, leading to resistance to anti-estrogenic drugs (117). IL-1β induces EMT by activating the IL-1β/IL-1RI/β-catenin pathway, thereby enabling TWIST1 to induce methylation of the *ESR1* promoter, which leads to reduced expression of ERα and thus increased tamoxifen resistance (118). Similarly, loss of demethylase also mediates tamoxifen resistance. Deletion of the DNA demethylase TET2 promotes the methylation of *ESR1*, thereby downregulating the expression of ERα and thus promoting tamoxifen resistance (119). Interestingly, ERβ showed the exact opposite effect to ERα in response to tamoxifen. In tamoxifen-resistant BC, ERβ is hypomethylated, suggesting that ERβ hypermethylation harms tamoxifen resistance (120).

Secondly, the methylation-mediated enhanced transcription of ERα-targeted genes can promote tamoxifen sensitivity, and vice versa. For example, ANCCA mediates the recruitment of MLL1 HMT at the promoters of ESR1 target genes for H3K4 methylation associated with gene activation, which may effectively induce tamoxifen sensitivity (121). Similarly, SETD1A is also involved in H3K4 methylation, subsequent ERα recruitment, and transcription of ERα target genes (122). Conversely, inhibition of *ESR1* transcription or ERα-mediated transcription promotes BC resistance to tamoxifen. Hypermethylation of estrogen response enhancers leads to reduced binding to ERα and downregulation of key regulators of ERα activity, resulting in weakened endocrine responses (123). By contrast, the hypomethylation of enhancers plays a role in the transformation of normal breast cells into endocrine-reactive BC (123). Overall, the methylation of the *ESR1* enhancer has potential in endocrine stratification therapy (123). Moreover, EZH2-mediated trimethylation of H3K27 promotes BC susceptibility (124). The single nucleotide polymorphism of EZH2 further promotes the methylation of H3K27, which inhibits the transcription, thereby reducing overall survival (OS) and progression-free survival in patients that are ER-positive/tamoxifen-treated (124). EZH2 may also promote the methylation of the *GREB1* promoter through DNMT1 and DNMT3B, thereby silencing *GREB1* and inhibiting ERα transcription, thus promoting tamoxifen resistance (125). Moreover, TET1 and TET3 promote demethylation of the *UCHL1* promoter, thereby promoting *UCHL1* transcription, which further downregulates ERα expression via the UCHL1-KLF5 axis, leading to tamoxifen resistance (126).

Finally, the methylation can also respond to tamoxifen through promoter methylation of ERα-associated genes. Among them, the upstream genes of ERα include *p21* (127), *WT1* (128) *ID4* (129) and *miR-27b* (130). Activation of p21 activates ERα transcription in ER-negative BC, thereby activating the ERα signaling pathway (129). LncRNA UCA1 interacts with the enhancer of *EZH2*, which inhibits p21 expression through H3K27 histone methylation on the *p21* promoter, thereby promoting tamoxifen resistance (131). In the development of tamoxifen resistance, WT1 is involved in the expression of ERα (128). Through RNA-seq and TCGA databases, Ren *et al* (132) found that *WT1* was hypermethylated and upregulated in all molecular subtypes of BC, which was closely related to the efficacy of tamoxifen in patients with BC. By contrast, ID4 inhibits ERα expression and regulates estrogen biosynthesis (129). In BC resistant to tamoxifen, ID4 is hypomethylated, suggesting that it may be the key to identifying drug resistance (133). However, there was no difference between ID4 hypermethylation and ID4 hypomethylation on the risk of disease progression (P=0.287) (133). As for miR-27b, it targets ERα to exert its anti-proliferation and anti-metastasis effects (130). The methylation of the *miR-27b* promoter promotes activation of HMGB3, leading to tamoxifen resistance (134). The downstream genes of ERα are relatively rich, including *NAT1* (135), *ELOVL2* (136) and *PRA* (137). High expression of ERα enhances the expression of *NAT1*, *ELOVL2* and *PRA*, so that the expression level of ERα is positively associated with *NAT1*, *ELOVL2* and *PRA* (135-137). The increased methylation of these gene promoters leads to decreased expression, which may lead to the dysfunction of ERα and induce tamoxifen resistance (138-143). Using methylation-specific PCR and bisulfite genomic sequencing, Kim *et al* (138) found that the methylation of *NAT1* was significantly enhanced in tamoxifen-resistant BC, while the methylation of *COMT*, *CYP1A1*, *CYP2D6* and *SULT1A1* was not significantly altered compared with the control group. Therefore, hypermethylation of *NAT1* may influence the initiation of tamoxifen resistance (138). LncRNA H19 mediates methylation of the *NAT1* promoter to downregulate NAT1 expression, which leads to tamoxifen resistance in BC (139). Similarly, the hypermethylation of the *ELOVL2* promoter and the *PRA* promoter downregulate *ELOVL2* and *PRA* to drive tamoxifen resistance (140,141). Notably, the majority (74%) of patients with *PRA* in PRA-negative BC did not exhibit methylation status (141). However, hypermethylation of downstream genes negatively associated with ERα expression also promotes tamoxifen resistance, such as *PAX2* (142) and *E-cadherin* (143). Estrogen receptors promote methylation of the *PAX2* promoter, thereby downregulating its expression (142). In patients with tamoxifen-resistant BC, abnormally elevated methylation of *PAX2* promoter leads to decreased expression of PAX2 mRNA (144). The estrogen/ERα signaling pathway downregulates E-cadherin to promote BC (143). The methylation of E-cadherin leads to the downregulation of E-cadherin expression, promoting the upregulation of Twist and the occurrence of EMT, thus resulting in tamoxifen resistance (145). In addition, although ERα upregulates the expression of MMP-1, the hypomethylation of the *MMP1* promoter leads to its overexpression, thereby inducing tamoxifen resistance in BC (146,147). As for ERβ, estrogen inhibits the expression of PTPRO through ERβ, thus inducing the occurrence of BC (148). The hypomethylation of the *PTPRO* promoter elevates its expression, thus promoting the sensitivity of tamoxifen (149).

Table II. Effect of PI3K/AKT/mTOR-related methylation on the tamoxifen response.

Gene[a]	Enzyme[b]	Type[c]	Number[d]	PI3K/AKT/mTOR[e]	Tamoxifen[f]	(Refs.)
PTEN	DNMT1	DNA promoter	NA	Up	Resistance	(151)
AKT	SETDB1	NA	NA	Up	Resistance	(153)
ERRFI1	NA	DNA promoter	NA	Up	Resistance	(157)
PITX2	NA	DNA promoter	NA	Up	Resistance	(158)
DOK7	NA	DNA promoter	NA	Up	Resistance	(159)
AK4	METTL3	RNA	m6A	Up	Resistance	(161)
SALL2	NA	DNA promoter	NA	Up	Resistance	(162)
ZDHHC22	NA	DNA promoter	NA	Up	Resistance	(163)
p16	EZH2	DNA promoter	NA	Up	Resistance	(166)
GDF15	NA	DNA promoter	NA	Up	Resistance	(167)
ATF3	YTHDF2	RNA	m6A	Up	Resistance	(169)
Beclin1	DNMT3B	DNA promoter	NA	Up	Resistance	(171)
TROP2	DNMT1	DNA promoter	NA	Down	Resistance	(173)
Ras	NA	H3K4me1	me1	Down	Sensitivity	(194)

[a]Target genes that are methylated or demethylated; [b]enzymes that mediate methylation or demethylation of target genes; [c]types of methylation, which mainly includes methylation on DNA promoters and methylation on lysine of histones; [d]the number of times the histone is methylated or the number of methyl groups on the lysine; [e]effects of targeted gene methylation on the function of PI3K/AKT/mTOR signaling pathway; [f]effects of targeted gene methylation on the tamoxifen response. PTEN, phosphatase and tensin homolog; ERRFI1, ERBB receptor feedback inhibitor 1; PITX2, paired-like homeodomain transcription factor 2; DOK7, downstream of kinase 7; AK4, adenylate kinase 4; SALL2, Sal-like protein 2; ZDHHC22, zinc finger DHHC-type containing 22; GDF15, Growth differentiation factor-1; ATF3, activating transcription factor-3; TROP2, tumor-associated calcium signal transducer 2; DNMT, DNA methyltransferase; SETDB1, SET domain bifurcated 1; METTL3, methyltransferase-like 3; EZH2, enhancer of zester homolog 2; YTHDF2, YTH domain-containing family 2; H3K4, histone H3 lysine 4; NA, not applicable

PI3K/AKT/mTOR. In addition to ERα, the methylation-mediated PI3K/AKT/mTOR signaling pathway and methylation on the PI3K/AKT/mTOR signaling pathway also influence tamoxifen resistance (12,14). In short, methylation-induced activation of the PI3K/AKT/mTOR signaling pathway contributes to tamoxifen resistance (Table II; Fig. 4) (14). By contrast, methylation-induced inactivation of the PI3K/AKT/mTOR signaling pathway promotes tamoxifen sensitivity (Table II; Fig. 4) (14).

Above all, the key proteins in the PI3K/AKT/mTOR signaling pathway (mainly including PTEN and AKT) can be methylated to reduce their expression, resulting in an altered response to tamoxifen. Low expression of *PTEN* due to hypermethylation of the *PTEN* promoter (-819 to -787 bp) predicts poor disease-free survival (DFS) and OS in patients with hormone-receptor-positive BC treated with tamoxifen (150). More precisely, in the -819 to -787 bp region of the promoter, only the hypermethylation of -796 CpG islands, but not the hypermethylation of the remaining four CpG islands, predicted shorter DFS and OS (150). A total of two sites in the *PTEN* promoter can be methylated by DNMT1, thereby downregulating the expression of *PTEN* and increasing phosphorylation of AKT, leading to tamoxifen resistance (151). By contrast, miR-146b reduced NF-κB-mediated MAT2A expression, which inhibited SAM-mediated methylation of the *PTEN* promoter to restore *PTEN* expression, thereby reversing tamoxifen resistance (152). Furthermore, SETDB1 regulates the expression of ER and AKT target genes by mediating the methylation and phosphorylation of AKT through interaction with PELP1, thus promoting tamoxifen resistance (153).

Next, the methylation of genes involved in inhibiting the PI3K/AKT/mTOR signaling pathway activates the PI3K/AKT/mTOR signaling pathway, thereby inducing tamoxifen resistance. Firstly, as for the AKT protein in PI3K/AKT/mTOR signaling pathway, ERRFI1 (154), PITX2 (155) and DOK7 (156) all can downregulate the expression of phosphorylated AKT, thereby inhibiting the PI3K/AKT/mTOR signaling pathway. The downregulated expression of ERRFI1 (157), PITX2 (158) and DOK7 (159) due to the hypermethylation of their promoters leads to impaired inhibition of the PI3K/AKT/mTOR signaling pathways, thereby inducing tamoxifen resistance in BC. Moreover, the silence of AK4 can downregulate the expression of p-AKT (160). METTL3-mediated increased methylation at multiple m6A sites of the 5'-UTR of AK4 mRNA stabilizes AK4 mRNA and thus increases ROS and p38 levels, leading to tamoxifen resistance (161). Secondly, inhibition of PTEN can also activate the PI3K/AKT/mTOR signaling pathway. In tamoxifen-resistant BCs, the *SALL2* promoter is hypermethylated, which inhibits *SALL2* expression and leads to *SALL2*-mediated transcription suppression of *ESR1* and *PTEN*, thus promoting the AKT/mTOR signaling pathway and leading to tamoxifen resistance (162). Thirdly, the activation of mTOR also ensures the function of PI3K/AKT/mTOR. The methylation of the *ZDHHC22* promoter leads to the low expression of ZDHHC22 in ER-negative BC, which leads to the activation of the mTOR, resulting in tamoxifen resistance (163). In addition, the reduction of p16 (164) and GDF15 (165) inhibits the occurrence and development of

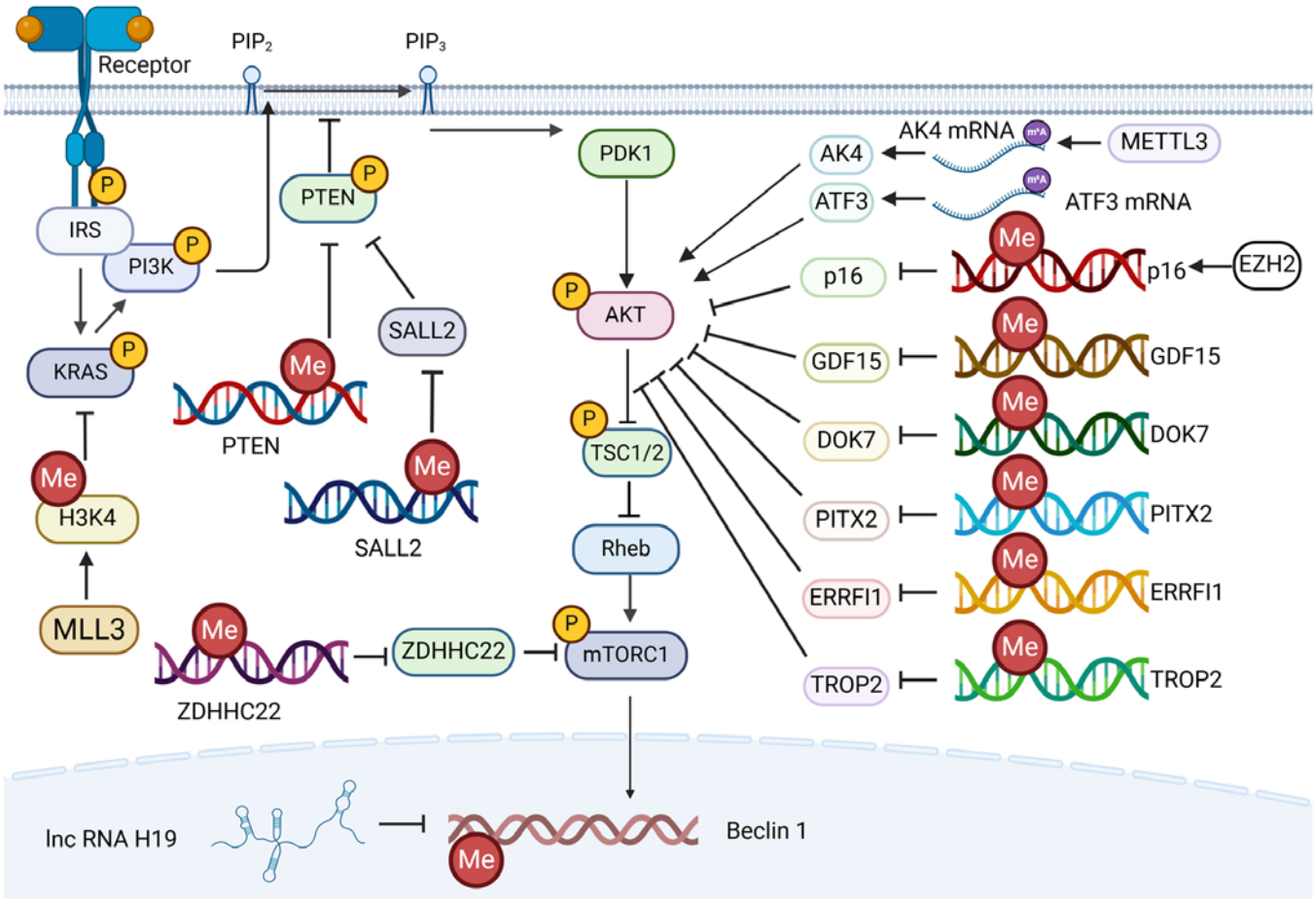

Figure 4. Effects of methylation of the PI3K/AKT/mTOR signaling pathway on tamoxifen. The methylation-induced activation of the PI3K/AKT/mTOR signaling pathway leads to tamoxifen resistance. PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; IRS, insulin receptor substrate; PTEN, phosphatase and tensin homolog; PDK1, 3-phosphoinositide-dependent protein kinase 1; TSC, tuberous sclerosis complex; mTORC1, target of rapamycin complex 1; H3K4, histone H3 lysine 4; MLL, mixed-lineage leukemia protein; SALL2, Sal-like protein 2; ZDHHC22, Zinc finger DHHC-type containing 22; METTL3, methyltransferase-like 3; AK4, adenylate kinase 4; ATF3, activating transcription factor-3; EZH2, enhancer of zester homolog 2; GDF15, Growth differentiation factor-1; DOK7, downstream of kinase 7; PITX2, paired-like homeodomain transcription factor 2; ERRFI1, ERBB receptor feedback inhibitor 1; TROP2, tumor-associated calcium signal transducer 2.

cancer through the inhibition of PI3K/AKT/mTOR signaling pathway. EZH2 downregulates p16 by promoting methylation of *p16*, which regulates the cell cycle and leads to tamoxifen resistance (166). In tamoxifen-resistant BC, the promoter of *GDF15* is hypermethylated, which leads to low expression and tamoxifen resistance (167).

After which, the hypomethylation of upstream promoter genes of the PI3K/AKT/mTOR signaling pathway promotes tamoxifen methylation via the PI3K/AKT/mTOR pathway. For example, ATF3 mediates the PI3K/AKT/mTOR signaling pathway by activating AKT phosphorylation, thereby increasing radiation resistance in BC (168). In BC, low expression of YTHDF2 leads to reduced hypomethylation of the 5'-UTR of ATF3 mRNA, resulting in the stabilization of ATF3 mRNA, which stimulates the expression of ABCB1 and leads to tamoxifen resistance (169). Similarly, the hypermethylation of downstream genes of the PI3K/AKT/mTOR signaling pathway also promotes tamoxifen resistance. The PI3K/AKT/mTOR signaling pathway regulates phagocytosis in macrophages through Beclin1 (170). LncRNA H19 binds and inhibits S-adenosylhomocysteine hydrolase, which inhibits DNMT3B binding to the *Beclin1* promoter and reduces *Beclin1* methylation, thus leading to autophagy dysregulation and tamoxifen resistance in BC (171).

Ultimately, the hypermethylation of upstream promoter genes of the PI3K/AKT/mTOR signaling pathway leads to tamoxifen resistance, which may be mediated by pathways other than the PI3K/AKT/mTOR signaling pathway. For instance, TROP2 promotes cell proliferation and migration through the PI3K/AKT/mTOR signaling pathway (172). However, in tamoxifen-resistant BC cells, the promoter of *TROP2* is methylated and silenced, which is meditated by DNMT1 (173).

Others. Except for ERα protein and PI3K/AKT/mTOR signaling pathways, the methylation of several other genes can influence BC sensitivity and resistance to tamoxifen. This can be divided into two components: i) Hypermethylation-mediated tamoxifen resistance (or hypomethylation-mediated tamoxifen sensitivity); and ii) hypomethylation-mediated tamoxifen resistance (or hypermethylation-mediated tamoxifen sensitivity).

For the first part, hypermethylation-mediated tamoxifen resistance mainly includes *VGLL4* (174), *DPYD* (175), *ZNF350* (176) and *MAGED1* (177). HAGLR inhibits *VGLL4*

Table III. Potential drugs for methylation on tamoxifen response.

Potential drug	Methylation[a]	Target[b]	Effect[c]	Tamoxifen[d]	Stage[e]	(Refs.)
Resveratrol, astragalus	Inhibit	*ESR1*	Promote *ESR1* expression	Sensitivity	Preclinical	(190)
Arsenic trioxide	Inhibit	*ESR1*	Promote *ESR1* expression	Sensitivity	Preclinical	(191)
5-aza-cdr	Inhibit	*ESR1*	Promote *ESR1* expression	Sensitivity	Preclinical	(192)
Sodium arsenate	Promote	*ESR1*, *BRCA1*	Inhibit *ESR1* and *BRCA1* expression	Resistance	Preclinical	(193)
Procainamide	Inhibit	*ERβ*	Promote *ERβ* expression	Resistance	Preclinical	(83)
Lycorine	Inhibit	*VGLL4*	Promote *VGLL4* expression	Sensitivity	Preclinical	(174)
Luteolin	Promote	H3K4	Inhibit *Ras* expression	Sensitivity	Preclinical	(194)

[a]Effects of potential drugs on methylation; [b]target genes that are methylated or demethylated; [c]effects of targeted genes being methylated; [d]effects of targeted gene methylation on tamoxifen response; [e]period in which potential drugs are in clinical application. ESR1, estrogen receptor 1; BRCA1, breast cancer susceptibility gene I; ERβ, estrogen receptor β; VGLL4, vestigial like family member 4; H3K4, histone H3 lysine 4.

expression by promoting DNMT1-mediated DNA hypermethylation, thereby promoting tamoxifen resistance (174). In tamoxifen-resistant BC cells, methylation of the *DPYD* promoter region leads to a decrease in DPYD mRNA (175). In addition, when the methylation inhibitor was applied in tamoxifen-resistant BC, the methylation of *ZNF350* and *MAGED1* promoters was significantly reduced, suggesting that *ZNF350* and *MAGED1* may play a role in tamoxifen resistance (176).

For the second part, hypomethylation-mediated tamoxifen resistance mainly includes *TSTD1* (177), *LDH* (178), *PAST1* (179) and *GNB4* (180). The hypomethylation of the *TSTD1* promoter leads to upregulation of *TSTD1*, which is associated with adverse reactions to tamoxifen therapy in patients with BC (177). In tamoxifen-treated cells, less methylation of the *LDH* promoter led to increased *LDH* expression, suggesting that *LDH* expression could promote tamoxifen resistance (178). Similarly, PSAT1 mRNA expression inhibited by hypermethylation of the *PSAT1* promoter predicts a good prognosis after tamoxifen treatment (179). Moreover, DNMT3B-mediated methylation of *GNB4* leads to silencing of *GNB4*, which promotes tamoxifen sensitivity (180).

5. Effects of tamoxifen on methylation

The degree of methylation is also altered after treatment with tamoxifen. However, there are relatively few studies on this area and they are relatively less comprehensive. Overall, tamoxifen reduces the level of methylation (109,181-183). Treatment of male rat embryos with tamoxifen resulted in increased methylation of multiple imprinted genes (such as Grb10, Igf2r, and Kcnq1), leading to downregulation of the expression of these imprinted genes (181). In BC, tamoxifen increases *CXCL12* expression through reducing the methylation of *CXCL12* promoters, thus making cells less susceptible to exogenous CXCL12 attraction to metastatic sites (178). In addition, tamoxifen altered the methylation of the *ESR1* promoter in patients with BC (182). Tamoxifen induces PRMT5 to translocate to nucleus, where it methylates the ERα protein, which causes corepressor proteins such as SMRT and HDAC1 to bind to the target gene promoter of ERα, thereby inhibiting transcription and cell proliferation (109). By contrast, in tamoxifen-resistant cell lines, PRMT5 is predominantly localized in the cytoplasm, suggesting that PRMT5 in the nucleus is a biomarker of tamoxifen sensitivity (109). For the embryonic development of sperm, the methylation of *IGF2-H19 ICR* in sperm is reduced after tamoxifen treatment, triggering the sperm to acquire paternal imprints and ensuring embryonic development (183). However, there was no significant increase or decrease in the overall methylation level of the rats (183).

It has been reported that after long-term tamoxifen treatment, the body may induce the formation of second tumors, especially EC, which may be related to tamoxifen-induced methylation impairment (Table III) (184-186). For example, the application of tamoxifen leads to the hypomethylation of the promoters of *CXCR4* and *CXCL12* by promoting the formation of DNA methyltransferase 3B4 splice variant, thereby up-regulating the expression of *CXCR4* and *CXCL12* in EC, thus promoting cell proliferation and metastasis (185). Moreover, tamoxifen induces hypomethylation of the *PAX2* promoter, thereby activating *PAX2* expression, which induces EC (186). In addition to this, the use of tamoxifen can also contribute to the development of liver cancer through methylation (187). Tryndyak *et al* (187) reported that the application of tamoxifen reduces the expression of DNMT1, DNMT3a and DNMT3b, which leads to the decrease of liver DNA methylation, thus inducing liver cancer.

6. Clinical application

Given the complex crosstalk between methylation and tamoxifen, it has multiple guidelines for clinical applications. Specifically, this can be divided into two parts, namely, the prediction of prognosis and recurrence of patients with BC treated with tamoxifen, and the exploration of potential clinical drugs.

Simply put, the higher degree of methylation, the worse prognosis of patients and the greater risk of relapse. Using the Illumina HumanMethylation450 BeadChip, Williams *et al* (176) found that tamoxifen-resistant cell lines share 3,000 hypermethylated and 200 hypomethylated CpG

islands. For example, the proportion of patients with PITX2 hypomethylation who did not have metastases after 10 years of tamoxifen treatment was higher than that of patients with PITX2 hypermethylation (158). Similarly, *DOK7* CpG is hypermethylated in leukocytes of patients which are tamoxifen-resistant, therefore the degree of DOK7 methylation is important for early diagnosis of tamoxifen resistance and prevention of cancer recurrence (159). Compared with the inactivation of genes due to promoter hypermethylation, the activation of growth-promoting genes due to promoter hypomethylation was also observed in tamoxifen-resistant cells (188). PSAT1 mRNA expression inhibited by hypermethylation of the *PSAT1* promoter predicts a good prognosis after tamoxifen treatment (179).

In addition, methylation of different promoters of some genes can result in different prognoses. The methylation of promoters in the U region of *GR* is associated with poorer OS, while methylation of promoters in the C region of *GR* is associated with improved OS (189). Thus, methylation of promoters in specific regions of *GR* can suggest a poor prognosis in patients who do not receive tamoxifen (189).

As for potential clinical drugs, the majority promote BC sensitivity to tamoxifen by affecting DNMT. The combination of resveratrol and astragalus leads to the downregulation of DNMT activity and decreases the methylation of the *ESR1* promoter, thus promoting the expression of ERα, which promotes tamoxifen sensitivity (190). Likewise, arsenic trioxide (191) and 5-aza-CdR (192) also inhibit DNMT1-mediated methylation of *ESR1*, thereby promoting ERα expression and thus tamoxifen sensitivity. However, sodium arsenate induces the recruitment of DNMT1, which increases CpG hypermethylation of *ESR1* and *BRCA1*, leading to tamoxifen resistance (193). In addition, procainamide promotes the expression of ERβ by inhibiting the methylation of the *ERβ* promoter, thereby inhibiting the signaling of ERα, thus inhibiting tamoxifen sensitivity (83). However, lycorine inhibits DNMT1-mediated methylation of *VGLL4*, thereby promoting VGLL4 expression and reversing tamoxifen resistance (174). In terms of the PI3K/AKT/mTOR signaling pathway, luteolin-induced MLL3 increases the mono-methylation of H3K4 on the *Ras* enhancer and promoter, thereby inhibiting *Ras* expression, which inhibits the activation of the PI3K/AKT/mTOR signaling pathway, thereby promoting tamoxifen sensitivity (194).

7. Discussion and conclusion

In summary, methylation promotes the development of tamoxifen resistance (115-118). The downregulation of ERα expression and abnormal activation of the PI3K/AKT/mTOR signaling pathway are the main causes of tamoxifen resistance by DNA methylation (10-14). By contrast, inhibition of methylation promotes BC sensitivity to tamoxifen (115-118). In addition, elevated methylation levels can be used as a predictive indicator of tamoxifen resistance (188-190). Moreover, a methylation inhibitor combined with tamoxifen is expected to improve the efficacy of tamoxifen (83,192-194).

Current research on methylation-induced changes in the efficacy of tamoxifen has focused on enhanced DNA methylation, which leads to the downregulation of the gene, resulting in tamoxifen resistance (18,144,173,195). However, methylation is not only limited to DNA methylation, but also m6A methylation, which is extremely important in RNA methylation and even protein methylation (196). In addition, methylation promotes tamoxifen resistance mainly by downregulating ERα and up-regulating the PI3K/AKT/mTOR signaling pathway (118,131,145,194). However, the ERα and PI3K/AKT/mTOR pathways are not isolated. Therefore, the crosstalk between them may have a further impact on tamoxifen resistance. ERα activates the PI3K/AKT/mTOR signaling pathway by downregulating PTEN expression and activating PTEN phosphorylation (197). The PI3K/AKT/mTOR signaling pathway acts as a bridge between growth factor and ERα signal transduction (198). However, the specific effect of tamoxifen, whether it increases resistance or sensitivity, needs more research.

Although drugs that target DNA methylation are gradually being used in the clinic and may provide new therapeutic approaches for improving tamoxifen response and cancer treatment, they are still at an early stage and there are numerous challenges to overcome. The precise interaction between methylation and tamoxifen resistance needs to be further elucidated, which will affect the accuracy of clinical applications. This includes controlling the selective effects of methyltransferases on target cells and the complex association between target gene methylation and the development of tamoxifen resistance. Although new generation sequencing can detect high-throughput methylation sites and accurately identify various DNA/RNA methylation patterns, it does not directly detect DNA methylation in body fluids, but relies more on DNA extraction and PCR techniques (199,200). In addition, inhibitors or potential drugs that inhibit the function or activity of DNMT also face challenges. Given that DNA methylation is present not only in tumor cells, but also in normal cells, it is a challenge to precisely target tumor cells during treatment with inhibitors or potential drugs targeting DNMT without affecting the epigenetic legacy modification of normal cells (201-203). Therefore, it is urgent to develop DNMT inhibitors that are selective for tumor cells and, more importantly, tamoxifen-resistant BC cells. Moreover, the combination of methylation-targeted drugs with endocrine therapy drugs such as tamoxifen is also an aspect worth considering. Compared with monotherapy, combination therapy can effectively reduce drug resistance (199). However, whether the combination therapy has a synergistic effect and whether it can minimize adverse reactions needs to be confirmed by further studies.

Furthermore, the present review only describes the crosstalk between methylation and tamoxifen. However, numerous other post-translational modifications can also affect tamoxifen resistance (184,204,205). Ubiquitination, acetylation and phosphorylation can also directly affect the ERα protein and the PI3K/AKT/mTOR signaling pathway, which may mediate the response to tamoxifen (184,204,205). Therefore, other post-translational modifications are worth investigation. In addition, the various post-translational modifications are not isolated, and they also have crosstalk with each other (206,207). For example, the methylation of histone is regulated by histone ubiquitination or by enzymes that catalyze ubiquitination (208). These modifications and their crosstalk play important roles in gene expression, genome stability, heterochromatin formation

and cancer development (208). Furthermore, HMTs and demethylases are phosphorylated, suggesting that phosphorylation can control the initiation and extent of histone methylation (209). Therefore, revealing the association between various modifications can not only improve the crosstalk between methylation and tamoxifen resistance, but also further improve the mechanism of tamoxifen resistance, providing a theoretical basis for improving the efficacy of tamoxifen.

Finally, tamoxifen is only one type of SERMs, and other endocrine drugs that are classified as SERMs have a similar mechanism to tamoxifen. Toremifene, raloxifene, opemifene, lasoxifene and bardoxifene are members of the SERM family (184). They are all able to interact with ER in specific tissues, resulting in conformational changes in the receptor, thus affecting the ERα-mediated signaling pathway (184). In theory, methylation-mediated downregulation of ERα could also promote resistance to these drugs. Unfortunately, this field of research is relatively rare and incomplete. Therefore, the research in related fields needs to be further explored. In addition, another class of drugs also targets the estrogen receptor, termed selective estrogen receptor degraders, and its representative drug is fulvestrant (210). Fulvestrant inhibits the binding of estrogen to ERα, but it promotes the ubiquitination degradation of ERα to inhibit ERα signaling (184). Similar to tamoxifen, loss of *ESR1* methylation leads to expression of *ESR1*, which restores sensitivity to fulvestrant (211). Although AKT inhibitors or PI3K inhibitors combined with fulvestrant are effective in ER-positive BC with palbociclib-resistance, it is not clear whether the PI3K/AKT/mTOR signaling pathway is one of the mechanisms of fulvestrant resistance (212). In addition, whether the activation of the PI3K/AKT/mTOR pathway or other signaling pathways promoted by methylation also leads to the development of resistance to fulvestrant remains to be further investigated. Furthermore, the present study that aimed to discuss the effect of methylation on tamoxifen resistance, mainly focused on preclinical studies, with phase I, II and III clinical trials practically absent. Therefore, future research may consider advancing the preclinical research into clinical practical application.

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Authors' contributions

HC conceived the study; JS and HC wrote the manuscript; JS, YH and SL collated the data; and HC revised and edited the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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