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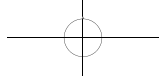
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A Review of the Relationship between Tea Drinking and Breast Cancer

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Abstract: The global incidence of breast cancer remains high and is increasing annually in some regions. Despite the variety of current treatments for breast cancer, the preventive and therapeutic effects are still limited due to the highly heterogeneous nature and complex biological mechanisms of breast cancer. In recent years, tea consumption has emerged as a research focus due to its possible anti-cancer properties. Numerous preclinical studies have demonstrated that regular tea intake could potentially curb the progression of breast cancer by influencing various biological mechanisms, including signaling pathways, cell cycle regulation, and immune system responses, among others. Nonetheless, the findings from epidemiological studies show considerable variability, and the connection between tea drinking and both the risk and outlook for breast cancer is shaped by numerous elements. These include the specific type of tea consumed, the quantity consumed, individual genetic variations, and environmental influences. This article summarizes the current research findings and delves into the connection between tea consumption and the risk as well as the prognosis of breast cancer among different regional populations. Meanwhile, it expounds on the potential molecular biological mechanisms behind it. The aim is to offer a theoretical foundation for the personalized prevention and treatment of breast cancer.

Keywords: Tea drinking; Breast cancer; Risk; Prognosis; Green tea; Black tea

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1. Introduction

Breast cancer stands as the most prevalent malignant tumor affecting women across the globe, with its incidence rising at a concerning pace. The latest statistics from the International Agency for Research on Cancer show that breast cancer continues to be the leading cause of cancer morbidity and mortality among women globally, with about 2.3 million new cases and 666,000 deaths in 2022 ^[1]. Breast cancer incidence is strongly associated with multiple factors, such as genetic elements, lifestyle choices, environmental exposures, and socioeconomic standing ^[2,3]. However, given the complexity of the etiology of breast cancer and its diverse clinical manifestations, its prevention and treatment still face many challenges.

In recent years, studies based on dietary patterns and lifestyle interventions have received widespread attention. Tea has become a research hotspot in this field due to its unique cultural value, rich bioactive components, and widespread global consumption. Based on different processing methods and sensory properties, tea is classified into six main types: green, white, yellow, oolong, red, and black tea ^[4]. Among them, black tea has the largest global production volume. Its main consumption areas include Europe, West Asia, and the United States. Green tea follows, ranking second in terms of production, and is mainly consumed in China and Japan. Oolong tea has a relatively lower production level and is predominantly consumed in the southeastern China and Taiwan region ^[5]. Tea is rich in various bioactive compounds, including polyphenols, amino acids, polysaccharides, and alkaloids, among others ^[6-8]. These active components endow tea with numerous beneficial qualities, including the ability to suppress bacteria, reduce inflammation, control blood sugar, regulate weight, and maintain cardiovascular health ^[9-11]. Numerous studies have shown the potential role of the active ingredients in tea in preventing and treating cancer ^[12-14], however, the results of related epidemiologic studies have not been agreed upon ^[15]. In the realm of breast cancer, the research results are equally inconsistent, and the link between tea intake and breast cancer remains ambiguous ^[16,17].

Consequently, the objective of this review is to fully combine the existing epidemiological findings with molecular biological processes in order to explore the intricate connection between tea intake and breast cancer, as well as to examine how the active compounds in tea influence the biological behavior of tumors, thereby providing new scientific insights into the prevention and management of breast cancer.

2. Association between tea consumption and breast cancer risk

2.1. Epidemiological research on tea intake and breast cancer risk across various regions

2.1.1. Europe

Before the 1990s, the epidemiological field on the association between tea intake and breast cancer risk was more limited, and the number of targeted studies was few. With the improvement of research methods and in-depth exploration of tea polyphenols and other components, the number of related studies is gradually increasing. In 1990, a Danish study on the link between dietary habits and the risk of breast cancer was conducted, analyzing questionnaires from 1,474 breast cancer patients. The findings indicated that tea intake was not significantly linked to the probability of developing breast cancer ^[18]. A forward-looking cohort study conducted in the Netherlands reached similar conclusions. The study focused on black tea and found that even after adjusting for confounding factors, groups that consumed tea more often (like five or more cups a day) had just a marginal uptick in the risk of breast cancer when compared to those who did not drink tea., but failed to achieve statistical significance (relative risk [RR] = 1.3; 95% confidence interval [CI]: 0.9–2.0). Further subgroup analysis considering vegetable and fruit intake likewise did not demonstrate any role of black tea consumption in reducing breast cancer risk ^[19]. A Swedish study based on a large breast cancer screening cohort similarly found no significant correlation between black tea intake and the incidence of breast cancer. The study included 1,271 breast cancer cases followed for nearly 10 years and demonstrated no meaningful correlation between tea consumption and the occurrence of breast cancer, either by general population, body mass index (BMI), or age. After adjusting for multiple covariates, there remained no statistically significant variation ^[20]. However, another Swedish study, centered around the Women's Lifestyle and Health (WLH) initiative, discovered that women who consumed more than one cup of tea daily faced a heightened risk of breast cancer when compared to those who did not drink tea (RR

= 1.19, 95% CI: 1.00–1.42)^[21]. A French cohort study additionally analyzed the effects of Herbal tea (mainly *Tilia cordata*, *Mentha piperita*, and *Verbena officinalis*) and showed that women who consumed more than 150 ml of Herbal tea per day had a significantly lower risk of breast cancer (RR = 0.43; 95% CI: 0.20–0.94; *P*-trend = 0.04). However, no noteworthy link was observed between the consumption of regular tea (including black or green tea) and the risk of breast cancer^[22]. It is essential to recognize that the study comprised only 95 breast cancer cases—a limited sample—and results should be viewed cautiously. Another French prospective cohort study followed 67,703 women for 11 years and ultimately included 2,868 cases of breast cancer. The findings indicated that tea intake did not have a meaningful correlation with breast cancer risk (*P* = 0.22). Although the cohort omitted details on tea varieties, the authors speculate that most participants drank traditional French black tea^[23].

2.1.2. America

In a cohort study of postmenopausal women in Iowa, no notable link was found between tea consumption and breast cancer risk (*P* = 0.28). Compared to those who do not consume tea, the RR for women drinking two or more cups daily was 1.14 (95% CI: 0.92–1.41)^[24]. However, only 9.5% of participants in the study reported drinking two or more cups of tea daily, which may limit the study's ability to detect potential associations. A major prospective cohort study utilizing the Nurses' Health Study II (NHS II) evaluated the link between tea, an important flavonol source, and breast cancer risk. The study participants showed an equal distribution in how often they consumed tea. No significant link was observed between tea intake (primarily black tea) and breast cancer risk over a follow-up period of nearly eight years. Compared to women consuming tea less than monthly, those drinking it two or more times daily showed a multivariable-adjusted hazard ratio of 1.02 (95% CI: 0.81–1.28; *P*-trend = 0.83)^[25]. Another NHS-based cohort study, despite 22 years of follow-up and analysis of 5,272 breast cancer cases from 11 states, likewise did not show a connection between the quantity of tea consumed and the risk of breast cancer (*P* = 0.25)^[26]. In addition, exploring a group of black women in the U.S. reached similar conclusions to the NHS cohort study^[27]. However, a trend indicating a negative correlation between the risk of breast cancer and the consumption of green tea (*P*-trend < 0.01), but no significant overall association with black tea (*P*-overall = 0.25), was noted in a cohort of women with a familial breast cancer background in the U.S. and Puerto Rico. In terms of tea consumption, consuming five or more weekly servings of green or black tea may reduce breast cancer risk^[28]. In a longitudinal study of Canadian females, no significant link was detected between tea consumption and the overall breast cancer risk (*P*-trend = 0.95). Statistically significant differences were also not observed across menopausal status, hormone replacement therapy, and BMI subgroups^[29]. A case-control study carried out in Uruguay, South America, aimed at examining the relationship between mate tea consumption and the risk of breast cancer, as well as evaluating its synergistic effects with dietary antioxidants. The research indicated that a high consumption of mate tea was notably linked to a lower risk of breast cancer, especially in those consuming more than 1 liter per day (odds ratio [OR] = 0.38 and OR = 0.41) and long-term users (OR = 0.62 for both models). Notably, the consumption of mate tea was not associated with dietary antioxidant levels. For conventional tea (which the study hypothesized was black tea), the negative association with breast cancer was only significant in people with high levels of dietary antioxidant intake^[30]. The impact of black tea and green tea on breast cancer has been investigated by researchers among Asian American groups, including women of Chinese, Japanese, and Filipino descent. The findings indicated that black tea intake had no significant correlation with breast cancer risk. In contrast, green tea consumption was linked to a notable decrease in the risk of developing breast cancer. This protective effect remained significant even after adjusting for diverse dietary and

other potential confounding factors, and breast cancer risk was further reduced with increasing green tea intake. In addition, the research also examined the relationship between consumption of green tea and soy products with breast cancer risk, showing that green tea's protective benefits were predominantly observed in individuals with low soy consumption ^[31].

2.1.3. Asia

A Japanese study pooled data from two prospective cohorts of 35,004 women, 222 of whom were diagnosed with breast cancer. The findings indicated no notable link between green or black tea intake and breast cancer occurrence. Unlike studies in Asian American populations, when stratified by soybean soup intake, women consuming five or more cups of green tea daily showed no significant difference in breast cancer risk compared to those drinking less than one cup daily, irrespective of soy soup consumption ^[32]. In their large-scale cohort study, Iwasaki *et al.* ^[33] refined green tea intake into nine dose groups (from < 1 cup/week to ≥ 10 cups/day), the results showed that no significant correlation with breast cancer risk was observed for either total green tea intake or grouping by tea type (Sencha, Bancha, and Genmaicha). In addition, studies of oolong tea and black tea also showed consistent results. Conversely, a case-control study of Japanese women found that green tea consumption was linked to a lower breast cancer risk, yet this was only evident in women who drank it 2–3 times a week (adjusted OR = 0.63; 95% CI: 0.43–0.93) ^[34]. Among Chinese Singaporean women, no statistically significant links were found between the consumption of green and black tea and breast cancer risk. To further validate the effect of gene-environment interaction on breast cancer risk, the researchers performed a stratified analysis based on angiotensin-converting enzyme (*ACE*) genotypes. The results found that the frequency of green tea consumption was significantly negatively associated with breast cancer risk (*P*-trend = 0.039) and had a dose-response relationship in women harboring high-activity *ACE* genotypes, whereas women harboring low-activity *ACE* genotypes did not show such an association. Green tea intake exhibited a statistically significant interaction with the *ACE* genotype in influencing breast cancer risk (*P*-interaction = 0.01), but this result was not observed in black tea ^[35]. Another study by the same team further evaluated the interaction of green tea intake with *MTHFR*/*TYMS* genotype and folate intake level on breast cancer risk. The findings indicated that among women with high-activity *MTHFR*/*TYMS* genotypes and lower folate intake, a significant inverse relationship was observed between green tea intake and breast cancer risk relative to other subgroups (OR = 0.44; 95% CI: 0.22–0.89) ^[36]. Both studies highlight the potential value of combining diet with an individual's genetic background in breast cancer prevention. A retrospective case-control study conducted in southeastern China found that green tea consumption was significantly associated with a reduced breast cancer risk when compared to women who did not drink tea. Those who consumed ≥ 750 g of dry tea annually, based on the amount of tea consumed, had a 39% lower risk of developing breast cancer than non-tea drinkers (adjusted OR = 0.61; 95% CI: 0.48–0.78). Breast cancer risk decreased with increasing duration and frequency of consumption, showing a clear dose-response relationship ^[37]. However, in a large prospective cohort study involving around half a million adults in China, no significant link was identified between tea intake and breast cancer risk in the researchers' findings (*P* = 0.267). The study covered 10 different geographic regions in China and included a total of 1,552 breast cancer cases ^[38].

While multiple epidemiological studies have investigated the link between tea intake and breast cancer risk, no consistent findings have been reached. Most studies show no significant correlation between tea intake and breast cancer risk, and results vary widely across geographic regions and populations. For instance, in Europe and the Americas, most studies have not identified a correlation between the intake of green or black tea and the

risk of breast cancer, whereas an increased association has been observed in studies conducted in Asia. However, there were also differences in results between different types of studies, such as cohort studies and case-control studies in Japan, from the same countries in the same region. This phenomenon may be related to the limitations of the studies. First, tea drinking preferences vary by region and cultural background, with black tea predominantly consumed in Europe and North America, mate tea in parts of South America, and more green tea in Asia. The biological mechanisms of action of the main active ingredients of different tea classes may have varying effects on breast cancer risk, which may lead to biased results. Some studies incorporated in this paper did not distinguish among tea categories, and homogenized analyses may dilute or confound the actual effects of specific tea categories on breast cancer risk. Secondly, although many studies have made substantial adjustments for various influencing factors, such as dietary habits and lifestyle behaviors, there are still potential factors that have not been measured or adequately considered, which could influence the precision and applicability of the findings. In addition, some studies suffered from self-reporting bias, measurement error of tea drinking frequency, multiple attribution, and lack of stratified analysis of breast cancer subtypes, which may mask potential associations among study subjects. Notably, studies of gene-environment interactions suggest that tea consumption may influence breast cancer development through interactions with individual genotypes, emphasizing the combined effects of dietary factors and genetic background and revealing that tea consumption may have the potential to personalize breast cancer prevention.

2.2. Impact of menopause and hormone receptor status on the link between tea intake and breast cancer risk

It is widely recognized that the onset and progression of breast cancer are strongly linked to menopausal and hormone receptor conditions. Green tea intake may affect estrogen metabolism or binding in different menopausal states, which in turn affects breast cancer risk ^[39]. Therefore, numerous studies have examined the association between tea intake and breast cancer risk across various menopausal statuses and hormone receptor profiles.

A study conducted using a Swedish mammography cohort revealed that black tea consumption was significantly linked to an increased risk of breast cancer overall, particularly for estrogen receptor-positive/progesterone receptor-positive (ER+/PR+) tumors (P -trend < 0.007). Compared with non-tea drinkers, women who drank ≥ 2 cups of tea per day had a 36% increased risk of ER+/PR+ tumors; the relationship remained unchanged regardless of menopausal status, hormone use in postmenopausal women, or BMI (P for interaction ≥ 0.10 for all) ^[40]. This is generally consistent with the conclusion of another study based on the WLH cohort above, which also found that among postmenopausal women, women who drank 1 cup of tea per day had a 24% increased risk of breast cancer (P -value = 0.007) ^[21]. Although the study did not specify the type of tea consumed, it was hypothesized that black tea was predominantly consumed, taking into account the context of the study and tea culture. However, in the French prospective study cohort, when analyzed based on menopause stage and hormonal receptor profile, no significant association was identified between black tea consumption and breast cancer risk ^[23]. Another multicenter European prospective cohort study evaluated the link between tea consumption and the risk of developing breast cancer in different menopausal states. The study involved 10 European countries with a mean follow-up of 11 years, and a total of 1,064 premenopausal and 9,134 postmenopausal breast cancers were diagnosed. Tea consumption does not appear to have a statistically significant impact on the risk of developing breast cancer, regardless of menopausal status. For premenopausal breast cancer, the adjusted hazard ratio (HR) associated with high tea consumption was 0.98 (95% CI: 0.77–1.26), while for postmenopausal breast cancer, it was 0.95 (95% CI:

0.88–1.03). Analyzed by hormone receptor status, again, no significant differences were found ^[41].

The impact of green tea was explored in a case-control study conducted in Shanghai, China, which included 3,454 cases and 3,474 individuals in the control group. The results showed that individuals who habitually drank green tea experienced a modest decrease in the risk of developing breast cancer (OR = 0.88; 95% CI: 0.79–0.98). In premenopausal women, the duration, frequency, and quantity of green tea intake were all linked to a lower likelihood of developing breast cancer. In postmenopausal women, the relationship was only associated with older age at initiation and lower intake ^[42]. A separate investigation examining a cohort of Chinese women in Hong Kong revealed that the risk of developing breast cancer was not influenced by the specific type of tea consumed (including green, black, oolong, and other teas), but rather depended on the menopausal status of the women and the hormone receptor status of the tumors. In premenopausal women, the intake of tea was linked to a lower likelihood of developing breast cancer (OR = 0.62; 95% CI: 0.40–0.97). Conversely, in postmenopausal women, tea consumption appeared to correlate with a heightened risk of the disease (OR = 1.40; 95% CI: 1.00–1.96). Similar trends were observed among green tea drinkers. Further stratified analysis based on ER status found that post-menopausal ER-green tea drinkers had the most increased risk of breast cancer (OR = 2.99; 95% CI: 1.26–7.11) ^[43]. A case-control study conducted in a hospital setting in Nagano Prefecture, Japan, concluded that there was no notable association between the intake of green tea and the risk of developing breast cancer. The study did not stratify analyses by menopausal status and did not obtain statistically significant results when stratified by different hormone receptor status ^[44]. In another prospective study based on public health centers in Japan, menopausal status had no significant effect on green tea and breast cancer risk ^[33].

Among U.S. women with a familial predisposition to breast cancer, the consumption of green tea was linked to a notably reduced risk of ER+ breast cancer. In particular, a 19% reduction in the risk of ER+ breast cancer (95% CI: 0.68–0.97) was observed among women who consumed at least five cups of green tea per week. No meaningful correlation was identified between green tea intake and the likelihood of developing ER- breast cancer. Among postmenopausal women, an enduring negative correlation was also observed between green tea intake and the risk of breast cancer, with the trend reaching statistical significance (*P*-trend < 0.01). However, no correlation was observed in black tea ^[28]. In a study focusing on African American women in the U.S., researchers observed a potential link between tea consumption and an increased risk of breast cancer among postmenopausal participants (incidence rate ratio [IRR] = 1.44, 95% CI: 0.89–2.34). However, this association fell short of achieving statistical significance. Further stratification by ER/PR status also showed no significant association ^[27]. Herbal tea, which contains flavonoids, quercetin, and kaempferol, is more commonly consumed in the Mexican region than green and black tea. Herbal tea demonstrated protective benefits in premenopausal females, especially when consumed less than 3 cups per week (OR = 0.41; 95% CI: 0.18–0.92). However, in women after menopause, the association was not significant ^[45]. In addition, a case-control study from Wisconsin, Massachusetts, and New Hampshire found that among women under 50 years of age, those who consumed at least three cups of tea each day had a 37% lower risk of breast cancer than non-tea drinkers (adjusted OR = 0.63, 95% CI: 0.44–0.89), and a significant trend indicating an increased effect with higher consumption was observed (*P*-trend = 0.01) ^[46].

Research data on the effects of menopause and ER/PR expression on tea intake and breast carcinoma risk are still limited, and published findings lack consistency. However, in some of the studies where associations have been observed, green tea consumption appears to show a potential correlation with reduced breast carcinoma risk among premenopausal individuals as well as ER+ women, such as the research conducted in Shanghai, China, and the U.S. Cohort Study on women with hereditary breast cancer risk. In contrast, black tea intake appears to be

linked to a heightened risk of breast cancer, particularly among postmenopausal women and those with ER+/PR+ subtypes, as evidenced by findings from the Swedish Mammography Cohort Study and the WLH Cohort Study. Findings from multiple cross-sectional studies suggest that green tea consumption might be negatively correlated with blood estrogen concentrations, while black tea consumption appears to show a positive correlation with blood estrogen levels ^[47], suggesting that tea's effect on endogenous hormones may be an important factor contributing to these differential outcomes. Yet, it is necessary to note that this trend in risk relationships has not been consistently confirmed in all studies. For example, neither the French prospective study cohort nor the multicenter European prospective cohort study detected a substantial link between the consumption of black tea and the risk of breast cancer. This may be related to differences in population characteristics, tea drinking habits, type and quality of tea, as well as study design and statistical methods across studies. Consequently, additional high-quality, large-sample, multicenter investigations are required to delve deeper into this intricate relationship.

3. Relationship between tea intake and breast cancer outcomes

3.1. Effect of tea consumption on survival and recurrence of breast cancer patients

Currently, limited epidemiological research exists on the impact of tea intake on breast cancer outcomes. In 1998, a Japanese investigation initially examined this connection and observed that an increased intake of green tea before diagnosis correlated with markedly better outcomes for individuals diagnosed with stage I and II breast cancer, although this effect was not seen in those with stage III disease ^[48]. A separate investigation conducted by Japan's Aichi Cancer Center revealed that drinking at least three cups of green tea each day prior to diagnosis was associated with a reduced likelihood of breast cancer recurrence (HR = 0.69; 95% CI = 0.47–1.00). However, this protective benefit was observed exclusively in patients diagnosed with stage I breast cancer ^[49]. Seely *et al.* ^[50] conducted a meta-analysis combining data from two studies, revealing a pooled RR of 0.75 (95% CI: 0.47–1.19; $P = 0.22$) for breast cancer recurrence across all stages. However, when diving into subgroup analysis, the findings painted a clearer picture: the pooled RR for stage I and II breast cancer dropped to 0.56 (95% CI: 0.38–0.83; $P = 0.004$). This suggests that green tea consumption might play a role in reducing the risk of recurrence, particularly in the early stages of breast cancer. A cohort study from Sweden analyzed the relationship between black tea consumption and breast cancer mortality and showed no significant correlation between black tea consumption and breast cancer-specific and total mortality. Moreover, this finding was not influenced by factors such as hormone receptor classification, cancer stage at the time of diagnosis, or tobacco usage (all $P > 0.05$) ^[51]. A study based on a US NHS cohort explored the relationship between tea intake and survival after breast cancer diagnosis. A total of 1,054 breast cancer-related and 2,501 overall deaths were documented over a follow-up period spanning up to 30 years. The findings indicated that drinking tea after a breast cancer diagnosis was linked to reduced overall mortality. Specifically, breast cancer patients who regularly consumed more than three cups of tea daily experienced a 26% lower risk of dying from any cause (HR = 0.74; 95% CI: 0.58–0.95; P -trend = 0.04). Nevertheless, no link was found between tea intake and mortality specifically related to breast cancer. Following a stratified analysis based on estrogen receptor status and molecular subtypes, the association between tea consumption and both overall and breast cancer-specific mortality lost its significance ^[52]. This indicates that increased tea intake might be linked to improved overall survival in breast cancer survivors. In another prospective cohort study involving patients diagnosed with triple-negative breast cancer, women who regularly consumed tea after diagnosis had not only a lower all-cause mortality rate (HR = 0.57; 95% CI: 0.34–

0.93), but also a lower recurrence/disease-specific mortality rate (HR = 0.54; 95% CI: 0.31–0.96). However, no interaction with menopausal status was observed^[53]. A study conducted in Guangzhou, China, examined the link between tea intake and survival rates prior to and following a breast cancer diagnosis. The results indicated that no significant link was detected between tea intake and progression-free survival, regardless of whether it was before or after diagnosis. However, when delving deeper into the analysis by tea consumption category, it was discovered that the consumption of oolong tea had no significant correlation with the risk of breast cancer progression (HR = 1.32; 95% CI: 0.60–2.90). On the flip side, other teas, primarily green teas, markedly reduced the likelihood of progression (HR = 0.52; 95% CI: 0.29–0.91). In addition, those who drank tea ≥ 7 times per week had a better prognosis (HR = 0.30; 95% CI: 0.11–0.84), but the concentration of tea intake was not linked to the progression risk^[54].

The link between tea intake and breast cancer outcomes is still unclear. ER and menopausal status seem to have little impact on their correlation, whereas the variety of tea consumed may play a role in this relationship. There may be an association between green tea intake and improved prognosis and reduced risk of breast cancer progression in patients with early-stage breast cancer. However, there are fewer relevant studies on black tea. The existing research did not identify a meaningful link between black tea consumption and breast cancer mortality, necessitating further investigation into its possible effects. In addition, most studies have focused primarily on the impact of tea consumption on patient survival after breast cancer diagnosis, and few studies have examined the impact of tea consumption prior to diagnosis. This somewhat limits a comprehensive assessment of the potential role of tea consumption in early intervention for breast cancer.

3.2. Effects of tea consumption on health and functional status of breast cancer patients

As the survival of breast cancer patients continues to lengthen, the assessment of their prognosis has gradually shifted from survival alone to a more comprehensive perspective. On a basis of traditional indicators such as survival and mortality, quality of life (QoL) has also been included as an important consideration^[55]. A multicenter cohort study assessed the association between tea consumption and QoL in breast cancer patients through patient-reported outcomes (PROs). The study found that at one year after diagnosis, patients in all groups experienced a decrease in overall QoL and an increase in fatigue and pain. However, tea intake was not found to have a meaningful association with QoL, anxiety, depression, fatigue, or pain reported by breast cancer patients. Plus, studies have not observed significant associations between tea consumption and breast cancer recurrence-free survival, distant disease-free survival, and overall survival^[56]. Thus, existing evidence indicates that tea intake does not seem to substantially enhance the survival or quality of life of individuals with breast cancer.

It has been shown that breast cancer treatment may have an impact on cognitive function^[57]. Common cognitive impairments include decreased memory, attention, information processing speed, and so on^[58]. Although cognitive impairment gradually resolves after cancer treatment, these cognitive dysfunctions may persist for some cancer survivors^[59]. A large prospective cohort study demonstrated that consistent tea intake was linked to better cognitive performance, especially in delayed memory ($P = 0.04$). Tea drinkers had a more significant improvement in delayed memory at 18 and 36 months post-diagnosis than non-tea drinkers, with a mean improvement score of 1.91 (compared to 1.43 for non-tea drinkers), which was of small to moderate clinical significance (Cohen's $d = 0.46$)^[60]. This suggests that tea consumption may contribute to cognitive recovery in breast cancer patients after treatment.

4. Potential anticancer mechanisms of tea drinking

Polyphenols are the key bioactive constituents in tea, mainly including flavonoids, flavonols, phenolic acids, and others^[61]. Tea polyphenols (TP), commonly known as catechins, are a class of flavonoids with the basic structure of α -phenylbenzopyranes, which can be mainly divided into the following four types: epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC), and epicatechin (EC)^[62]. Due to the rich content of EGCG in tea and the numerous related studies, this paper will focus on the anticancer mechanism of EGCG and cover the related contents of other components.

4.1. Inhibition of signaling pathways and oxidative stress regulation

Approximately 15–20% of breast cancer patients exhibit *HER-2* gene overexpression, and this abnormal expression is typically strongly associated with poor patient prognosis^[63]. EGCG can inhibit the phosphorylation of HER-2, which in turn inhibits the activation of Stat3 as well as the promoter activity of c-fos and cyclin D1, and decreases the intracellular cyclin D1 and Bcl-XL protein levels, thereby inhibiting the growth of HER-2 positive breast cancer cells. In addition, EGCG enhanced the inhibitory effect of paclitaxel on such breast cancer cells^[64]. In ER+/PR+ breast cancer cells, EGCG can also exert the same inhibitory effect. It promotes the separation of Hsp90 from progesterone receptor B (PR-B) by activating the p38MAPK/CK2 signaling pathway, leading to the translocation of PR-B to the nucleus and the recruitment of NCoR and HDAC1 co-repressor complexes at the half-progestin-responsive element site on the ER α promoter, which inhibits the transcriptional activity and expression level of the *ER α* gene. In addition, EGCG significantly inhibited ER α -associated genomic and non-genomic signaling pathways^[65]. HBP1 is a transcriptional repressor that inhibits the transcriptional activation of the Wnt signaling pathway, and its mutation is associated with the proliferation of breast cancer cells. EGCG can increase the expression level of HBP1 protein by increasing the stability of HBP1 mRNA, inhibit the activity of the Wnt signaling pathway, and reduce the proliferation and invasiveness of breast cancer cells^[66]. Proline dehydrogenase (PRODH) is a key enzyme involved in proline metabolism within cells and supports cancer cell survival and growth by promoting proline metabolism. EGCG significantly suppresses the expression of PRODH and its regulatory proteins in triple-negative breast cancer (TNBC) cell lines. Similarly, in patient-derived xenograft mouse models, EGCG markedly downregulates PRODH expression and inhibits tumor growth^[67]. Kaempferol (Kaem) is a natural flavonoid phytoestrogen widely found in tea and other plants. Triclosan (TCS), a synthetic antimicrobial agent with estrogenic activity, promotes the proliferation of MCF-7 breast cancer cells through activation of the non-genomic ER signaling pathway associated with IGF-1R. Kaem can significantly impede the growth of cancer cells caused by TCS or induced 17 β -estradiol (E2) by regulating the expression of cell cycle-related genes (such as the down-regulation of cyclin D1 and cyclin E) as well as apoptosis-related genes (such as the up-regulation of Bax). In a xenograft breast cancer mouse model, Kaem also significantly inhibited tumor growth induced by E2 or TCS. This suggests that Kaem has the potential to mitigate the risk of breast cancer triggered by endogenous and exogenous estrogens by inhibiting ER and IGF-1R signaling pathways^[68].

Green tea catechins (GTCs), the primary bioactive compounds in green tea, may help prevent carcinogenesis by reducing oxidative stress-induced processes. Low-dose exposure to environmental carcinogens triggers an increase in reactive oxygen species (ROS) levels in mammary cells, which activates the ERK pathway, resulting in cell growth and DNA impairment. GTCs significantly inhibit this process at non-cytotoxic concentrations and continue to exert their effects under prolonged exposure conditions, as well as preventing cells from gaining cancer-related traits, including decreased reliance on growth factors, the ability to grow independently of

anchorage, and enhanced migratory ability^[69]. Green tea extract (GTE) also possesses antioxidant activity, which effectively scavenges free radicals at low concentrations, significantly reduces H₂O₂-induced ROS levels, and inhibits serum MMP-2 and MMP-9 activities in breast cancer patients in a concentration-dependent manner. It offers additional evidence supporting tumor progression inhibition via antioxidant mechanisms^[70].

4.2. Suppression of tumor microenvironment and angiogenesis

It was found that TNBC cell-secreted factors can induce adipose-derived mesenchymal stem/stromal cells (ADMSC) to exhibit inflammatory and cancer-associated adipocyte (CAA)-like phenotypes, which were characterized by the increased expression of CAA-associated cytokines (such as CCL2, CCL5, etc.) and immunomodulatory factors (such as COX2, HIF-1 α , etc.), and the expression of Snail was upregulated. EGCG could effectively inhibit the *CAA* gene expression induced by TNBC cell secretion factors, as well as the activation state of Smad2 and NF- κ B. This suggests that EGCG is able to prevent the inflammatory response and the formation of CAA-like phenotype in ADMSC triggered by TNBC cell-secreted factors^[71].

Tumor growth relies on angiogenesis, a process primarily stimulated by vascular endothelial growth factor (VEGF), a key mediator of angiogenesis^[72]. A previous study found that both GTE and its single catechin component effectively restricted the growth of breast cancer cells and vascular endothelial cells and reduced tumor vessel density in mice^[73]. Further studies showed that GTE and EGCG could reduce VEGF secretion in breast cancer cells and endothelial cells by inhibiting VEGF promoter activity and RNA transcription. GTE may also be involved in the inhibitory effect on VEGF by decreasing the transcription of c-fos and c-jun RNA and the expression of protein kinase C^[74]. Polyphenon E (PolyE), a standardized green tea extract, can block STAT3 activation by suppressing STAT3 phosphorylation and its dimerization with STAT1. This mechanism downregulates the expression levels of VEGF and MMP-9, thereby inhibiting angiogenesis and breast cancer cell migration^[75]. Fibroblast growth factors (FGFs) also play a key role in tumor angiogenesis and tumor growth. gTE and EGCG not only reduce the transcriptional levels of acidic and basic fibroblast growth factor (bFGF) in endothelial cells and breast cancer cells, but also decrease the bFGF protein levels in cells in a dose-dependent manner^[76].

4.3. Suppression of cancer cell invasion and metastasis

Epithelial-mesenchymal transition (EMT) is a key process that promotes cancer cell invasion and metastasis. Studies have shown that EGCG can inhibit EMT in breast cancer cells by activating FOXO3a, which induces an increase in the expression of ER α , and then up-regulates epithelial marker genes (such as E-cadherin and MTA3) and down-regulates mesenchymal marker genes (such as Snail)^[77]. Matrix metalloproteinases (MMPs), primarily responsible for breaking down the extracellular matrix, are crucial in tumor progression and metastasis. Both TP and EGCG significantly downregulate *MMP-9* gene expression and enzyme activity to inhibit cellular matrix degradation. They can also diminish cytoplasmic and nuclear β -catenin buildup by decreasing AKT phosphorylation levels, thereby down-regulating genes regulated by β -catenin/Tcf (e.g., c-myc and AKT1) and further inhibiting the proliferation and invasion of tumor cells^[78]. For the enhanced MMP-9 expression and activity induced by epidermal growth factor (EGF), EGCG could significantly reduce the expression and activity of MMP-9 by blocking the phosphorylation of FAK, PI3K, and ERK, which in turn inhibited the binding of NF- κ B and AP-1 transcription factors to the MMP-9 promoter. Not only that, EGCG also inhibited the interaction of integrin α 5 β 1 with extracellular matrix fibronectin, further weakening the synergistic induction of MMP-

9 overexpression by EGF and fibronectin ^[79]. In addition, EGCG has an inhibitory effect on the Rac1 pathway, which decreases VASP expression and thus suppresses the migration and invasion capabilities of MCF-7 cells ^[80].

4.4. Inhibition of cellular metabolic mechanisms

The expression of fatty acid synthase (FAS) is significantly upregulated in cancer cells compared to normal cells, and inhibition of FAS expression has been shown to selectively inhibit cancer cell growth ^[81]. Studies have shown that extracts from both green and black tea, especially EGCG and TF-3 therein, significantly inhibit the protein and mRNA expression of FAS. Both can block PI3K/Akt signaling pathway activation by inhibiting EGF binding to EGFR, thereby impairing the binding capacity of nuclear transcription factor Sp-1 to DNA. Since Sp-1 cannot effectively bind to the promoter region of the *FAS* gene, this process inhibits the abnormally high expression of FAS in the MCF-7 breast cancer cell line, exerting its potential anticancer and lipid-lowering effects ^[82]. In addition, the glycolytic pathway occupies an important position in the metabolism of cancer cells. EGCG can significantly inhibit the glycolytic process by decreasing the activity and mRNA expression of key enzymes of glycolysis (such as hexokinase), as well as by decreasing the expression of HIF1 α and GLUT1, thereby decreasing the uptake of glucose and the synthesis of ATP ^[83].

4.5. Enhancement of anti-tumor immunomodulation

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous class of myeloid cells that can inhibit the function of T cells through a variety of mechanisms, thereby promoting tumor growth and metastasis. EGCG was found to significantly inhibit the proliferation of 4T1 breast cancer cells. In *in vivo* experiments, EGCG notably diminished the buildup of MDSCs in the peripheral blood, spleen, and tumor tissues of 4T1 tumor-bearing mice and increased the proportion of CD4⁺ and CD8⁺ T cells, thus enhancing anti-tumor immune responses. In an *in vitro* assay, EGCG effectively inhibited the survival and proliferation of MDSCs by down-regulating classical signaling pathways (such as Arg-1/iNOS/Nox2/NF- κ B/STAT3) as well as regulating non-classical pathways (such as ECM-receptor interactions and adhesion patch formation) in MDSCs. Meanwhile, the expression of nine key genes in MDSCs was restored. This provides new insights into the role of EGCG in anti-tumor immunity ^[84].

4.6. Impact on epigenetic regulation

Tissue inhibitors of matrix metalloproteinases (TIMPs) are a group of naturally occurring proteins whose main function is to inhibit the activity of MMPs. GTP and EGCG can significantly induce TIMP-3 expression in breast cancer cells through epigenetic pathways, thereby inhibiting the activity of MMP-2 and MMP-9 and decreasing the ability of cell invasion. Specifically, GTP and EGCG reduce the modification of H3K27 trimethylation in the TIMP-3 promoter region and increase the level of H3K9/18 acetylation by decreasing the protein expression of EZH2 and class I HDACs, which in turn induces the expression of TIMP-3 and restores the balance between MMP and TIMP ^[85]. Signal peptide-CUB-EGF domain-containing protein 2 (SCUBE2) is an oncogene whose expression is usually down-regulated in tumor tissues, and one of the main reasons for the down-regulation is the hypermethylation of its promoter region. It was found that EGCG could significantly upregulate SCUBE2 by decreasing the methylation of the SCUBE2 promoter through decreasing the activity of DNA methyltransferase (DNMT), while enhancing E-cadherin levels and reducing vimentin levels, thereby suppressing the migration and invasion of breast cancer cells ^[86].

4.7. Cell cycle arrest and pro-apoptotic regulation

Growth factors regulate normal cell cycle protein homeostasis and promote cell progression from G1 to S phase, a process that involves accumulation of cell cycle proteins, reduction of CDK inhibitors, activation of CDK, and phosphorylation of pRB^[87]. EGCG can inhibit cyclin D1-associated pRB kinase activity and its phosphorylation by inducing the expression of p21(CIP1/WAF1/SDI1), thereby inhibiting the entry of MCF10A mammary epithelial cells into the S-phase under the stimulation of EGF, which in turn affects the progression of the cell cycle. Notably, the inducing effect of EGCG on p21 was dependent on EGF signaling, which increased p21 expression approximately three-fold in the presence of EGF, but no notable effect was observed in the absence of EGF^[88].

EGC, which is structurally similar to EGCG, inhibits breast cancer cell growth by inducing apoptosis, has no effect on cell cycle progression, and has no impact on the proliferation of healthy mammary epithelial cells. EGC-induced apoptosis involves the Fas signaling pathway and correlates with a decrease in the levels of the Bcl-2 protein and an increase in the levels of the Bax protein^[89]. Telomerase, a reverse transcriptase that maintains the integrity of chromosome ends (telomeres), is activated in more than 90% of breast cancers, and its increased activity correlates with a poor prognosis^[90]. EGCG can inhibit cell proliferation and induce apoptosis by inhibiting mRNA and protein expression of telomerase reverse transcriptase and down-regulating telomerase activity in MCF-7 breast cancer cell lines^[91]. Survivin belongs to the family of apoptosis-inhibiting proteins, which have the function of inhibiting apoptosis as well as regulating cell division. TP can induce apoptosis in breast cancer cells by down-regulating survivin expression. In a nude mouse transplantation tumor model, TP significantly reduced the volume of the tumor and the expression level of survivin protein in tumor tissues^[92]. As the main active component of TP, EGCG can inhibit survivin promoter activity by suppressing the AKT signaling pathway, leading to significant down-regulation of survivin mRNA and protein levels. This process further activates caspase-9, which ultimately induces apoptosis in breast cancer cells^[93]. The pro-apoptotic effects of theaflavins were more pronounced in breast cancer cells expressing functional p53. Specifically, theaflavins promote the translocation of Bax to mitochondria by up-regulating the expression of p53 and the pro-apoptotic protein Bax, resulting in the disruption of mitochondrial membrane potential, cytochrome c release, caspase cascade activation, and subsequent apoptosis induction^[94]. This mechanism is similar to the anti-tumor mechanism of Ziyang tea polyphenol extract (ZTP). In addition to this, ZTP-treated MCF-7 cells produce excessive ROS, which play a key role in ZTP-induced apoptosis^[95]. ZIP9 (SLC39A9) is a membrane androgen receptor that induces apoptosis upon binding to androgen. Studies have shown that both (-)-epicatechin and (+)-catechin have a high affinity for ZIP9, but their mechanisms of action are different: (-)-epicatechin exhibits significant agonist activity at low concentrations, whereas (+)-catechin exhibits antagonistic effects. In MDA-MB-468 breast cancer cells, (-)-epicatechin induces apoptosis by activating the ZIP9 signaling pathway, decreasing intracellular cAMP production, and increasing free zinc levels^[96]. Deoxycytidine triphosphate deaminase (DCTD) is an enzyme involved in DNA metabolism that indirectly affects DNA synthesis and repair, mainly by regulating the balance of the nucleotide pool. Teadenol B, a chemical derived from microbial fermented tea, is able to inhibit the growth of cancer cells by inducing early and late apoptosis of MDA-MB-231 cells. In MDA-MB-231 cells, the transformation of autophagy marker LC3-I to LC3-II was significantly increased after teadenol B treatment, while the level of SQSTM protein was decreased, suggesting that teadenol B has a significant autophagy induction effect^[97].

Besides, the dose of EGCG determined the pattern of MCF-7 cell death: low-dose (10–50 μ M) EGCG induced apoptosis by increasing ROS generation, activating c-Jun N-terminal kinase and caspase-9/3, decreasing

mitochondrial membrane potential, and increasing the Bax/Bcl-2 ratio; whereas, high-dose (100–400 μM) EGCG mainly induced cell necrosis by decreasing intracellular ATP levels^[98].

4.8. Improving drug resistance

Multidrug resistance (MDR) is one of the common and unresolved challenges in the treatment of malignant tumors and has several manifestations. One of these manifestations is the overexpression of p-glycoprotein (Pgp), which decreases the intracellular concentration of a drug by mediating the transport of the drug to the extracellular compartment and expelling the chemotherapeutic agent out of the cell^[99]. TP was able to reverse MDR by inhibiting the activity of Pgp. Assessment of Pgp activity can be accomplished by assaying cellular uptake of the Pgp substrate ^{99m}Tc-tetrofosmin. At a concentration of 500 $\mu\text{g/mL}$, TP increased the uptake of ^{99m}Tc-tetrofosmin by adriamycin-resistant cell line MCF-7/Adr cells by 16-fold, whereas the conventional MDR modulator, quinidine, increased by only 4-fold at a concentration of 200 μM , suggesting that TP has a significant potential for the reversal of Pgp-mediated resistance^[100]. In terms of tamoxifen resistance, EGCG inhibits ERK signaling pathway activity by reducing EGFR expression and phosphorylation levels, leading to reduced cell proliferation and invasion. In addition, EGCG can significantly reduce the expression of matrix metalloproteinases (MMP-2 and MMP-9) and its inducer EMMPRIN, increase the level of tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), inhibit the activity of MMP, and multi-target the invasion and metastasis ability of tamoxifen-resistant breast cancer cells^[101].

4.9. Synergistic mechanism

Through *in vivo* and *in vitro* experimental studies, the combined use of GTE and tamoxifen has a synergistic anticancer effect on breast cancer. GTE can enhance the antitumor activity of tamoxifen by down-regulating ER α expression, blocking ER-dependent transcription, and inhibiting estrogen-induced MAPK signaling pathways. This combined therapy more effectively suppresses breast cancer cell growth and enhances apoptosis compared to either drug individually^[102]. The combination of EGCG and HDAC inhibitors significantly enhanced ER α reactivation, making ER α -breast cancer cells sensitive to estradiol and tamoxifen. This effect activates ER α transcription primarily by modulating the histone acetylation and methylation status of the ER α promoter and decreasing the binding of the transcriptional repressor complex in this region, thus providing a potential therapeutic strategy for hormone-resistant breast cancer^[103]. 5-aza-20-deoxycytidine (5-aza 20 dC) is a DNA methyltransferase inhibitor with the ability to induce DNA demethylation, but it can trigger cytotoxicity at higher doses. Combination therapy with low concentrations of EGCG and 5-aza 20 dC significantly inhibited breast cancer cell growth, with better results than monotherapy and no significant toxicity to normal breast epithelial cells. The combination therapy enhanced cell cycle arrest and apoptosis by down-regulating cycle-related genes and anti-apoptotic genes and up-regulating pro-apoptotic genes. In addition, this combination treatment reduced the expression of DNMT1, DNMT3b, and HDAC1, inducing DNA hypomethylation and histone modification changes^[104]. A similar epigenetic regulatory mechanism was observed when GTP was combined with thiosulfonamide in broccoli buds^[105]. Although the anti-angiogenic drug sunitinib has shown significant efficacy in the treatment of many cancers, it suffers from drug resistance and a narrow therapeutic window. It was found that the combination of EGCG and sunitinib could reduce VEGF secretion while down-regulating IRS-1 levels and inhibiting the MAPK signaling pathway. This combination can effectively reduce tumor size and inhibit tumor angiogenesis, thus enhancing the anti-tumor effect of sunitinib^[106].

5. Conclusion

Although preclinical studies provide some theoretical support for the potential of tea consumption in breast cancer prevention and treatment, the results of epidemiologic studies still fail to draw consistent conclusions. This discrepancy may be closely related to the complex interplay of tea-drinking habits, tea types, genetic diversity, and multiple confounding factors. Future studies should pay more attention to the heterogeneity of different populations and explore the biological mechanisms of various components of tea. Through a comprehensive analysis of molecular biology, genetics, and epigenetics, the exact function and potential mechanisms of tea intake in preventing and treating breast cancer were additionally explored to provide a more accurate scientific basis for the personalized prevention and treatment of breast cancer.

Disclosure statement

The authors declare no conflict of interest.

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Construction of an Evidence-based Practice Protocol for Perioperative Nutritional Optimization in Esophageal Cancer Patients

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Abstract: *Objective:* To construct an evidence-based practice plan for perioperative nutritional optimization in esophageal cancer patients. *Methods:* By systematically searching relevant guidelines at home and abroad, two experts independently assessed the quality of the guidelines, extracted valuable evidence and recommendations, and initially formed a draft nursing program. Subsequently, an expert panel was organized to conduct a detailed discussion to review the practicality and effectiveness of the recommendations one by one, and the program was finally revised and improved. *Results:* The protocol covered four stages of patients' admission, preoperative, postoperative, and discharge, involving specific contents such as nutritional assessment, risk screening, dysphagia assessment, nutritional therapy, enteral and parenteral nutritional support, symptom management, and health education. The program included a total of 61 entries, with 33 class A recommendations and 28 class B recommendations. *Conclusion:* The constructed perioperative nutritional care program for esophageal cancer patients is scientific and practical, and can provide practical guidance for clinical care.

Keywords: Esophageal cancer; Perioperative care; Nutritional care; Evidence-based care; Assessment; Health education; Complications

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1. Introduction

Esophageal cancer is a common malignant tumor of the digestive system. With advancements in modern medicine, including surgery, radiotherapy, chemotherapy, and other treatments, the survival rate of patients has gradually improved^[1]. However, due to multiple complications during surgery and treatment, patients still face many challenges in perioperative management, especially poor nutritional status in the perioperative period, which has a crucial impact on surgical outcome, postoperative recovery, and quality of life, and not only exacerbates postoperative complications and prolongs hospitalization, but also leads to a delay in the recovery process^[2]. Relevant studies have shown that early nutritional screening and assessment of esophageal cancer patients and

timely implementation of nutritional intervention can effectively improve the nutritional status of patients, reduce the occurrence of postoperative complications, and thus improve the prognostic effect and accelerate the recovery process^[3]. However, at present, there is a lack of systematic and standardized practice protocols for perioperative nutritional intervention in esophageal cancer patients, and many clinical caregivers lack the guidance of evidence-based bases, which leads to a certain degree of variability in care outcomes. Evidence-based medicine, as a medical practice model based on the best evidence, helps clinical workers make scientific and rational decisions by collecting, evaluating, and applying high-quality evidence from clinical studies^[4]. This study aims to construct a set of evidence-based perioperative nutritional optimization practice protocols for patients with esophageal cancer, by systematically evaluating the existing nutritional care guidelines at home and abroad, refining the effective evidence, and combining the current scientific research results with the actual clinical needs, to form a set of comprehensive, scientific, and practical care protocols, which are designed to provide more standardized guidance for perioperative care of patients with esophageal cancer.

2. Information and methodology

2.1. Establishment of an evidence-based practice program building team

A special working group was set up, consisting of one postgraduate supervisor, two nurse leaders, and three current master's degree students, all of whom had received training in evidence-based nursing systems and possessed a certain degree of theoretical foundation and practical experience. The team members jointly participated in the search for relevant nursing guidelines, quality assessment, and integration of recommendations, and finally formed a preliminary draft protocol for perioperative nutritional care of esophageal cancer patients. During the program validation stage, an expert panel was formed consisting of one chief physician, two deputy chief physicians, one dietitian, three charge nurses, and one nursing backbone (case nutrition nurse). The eight expert panel members had an age range of 31–54 years old, with an average age of 40.35 ± 3.23 years old, and had extensive experience in the work. The working experience ranged from 9–25 years. Academic background: four undergraduates, three masters, and one PhD. The multidisciplinary cooperation and expert participation characteristics of the group ensured the quality and authority of the evidence-based practice program and laid a solid foundation for subsequent program revision and promotion.

2.2. Research methodology

2.2.1. Guide search

In this study, we searched domestic and international authoritative databases and the official websites of relevant nutritional societies to collect guidelines related to the nutritional management of esophageal cancer patients. The search was performed using the terms “esophageal neoplasm/esophagus cancer,” “nutrition/nutritional,” “malnutrition/malnourished,” “parenteral nutrition,” “enteral nutrition,” “esophagectomy/esophageal resection/esophagus resection,” “guideline,” and other keywords in English, and Chinese keywords such as “parenteral nutrition,” “esophagectomy,” and “guidelines” were searched with a combination of subject terms and free words. The search timeframe was set from January 1, 2016, to May 1, 2024, to ensure that the data were up-to-date and authoritative. Databases were selected from PubMed, Cochrane Library, OVID, EMBASE, CINAHL, SinoMed, China Knowledge Network (CNKI), Wipo, Wanfang, and others. Relevant guidelines were also sourced from the Medical Pulse Guidelines Network, World Health Organization (WHO), JBI Evidence-Based Library,

National Institute for Clinical Excellence (NICE) in the UK, Scottish Intercollegiate Guidelines Network (SIGN), Registered Nurses Association of Ontario (RNAO) in Canada, Canadian Clinical Practice Guidelines Database (CMACPGInfobase), and the New Zealand Guidelines Study Group (NZGG) ^[5]. In addition, websites of important organizations in the field of nutrition were searched, including the European Society for Parenteral and Enteral Nutrition (ESPEN), the American Society for Parenteral and Enteral Nutrition (ASPEN), the American Dietitian Nutritionist Association (AND), and the official website of the Chinese Society of Nutrition, to ensure that the guidelines were comprehensive and authoritative.

2.2.2. Guideline inclusion and exclusion criteria

Inclusion criteria: (1) Guidelines issued by authoritative international or domestic medical organizations, societies, governmental health agencies, or professional associations; (2) Guidelines cover perioperative nutritional management of esophageal cancer patients, including nutritional assessment, screening, nutritional interventions, and nutritional supportive measures; (3) Guidelines are formulated based on systematic reviews, randomized controlled trials, or other high-quality clinical research evidence, and have a clear grade of recommendation; (4) The guidelines were published or updated between 1 January 2016 and 1 May 2024 to ensure the timeliness of the content; (5) Published in Chinese or English for easy understanding and application by researchers.

Exclusion criteria: (1) Guidelines, consensus or review articles written by individuals or non-official organizations are not authoritative; (2) Guidelines do not cover perioperative nutritional management of esophageal cancer or are mainly for patients with other diseases; (3) Guidelines rely only on the opinions of experts or clinical experience, and are not based on systematic reviews or high-quality research evidence, with insufficient evidence-based support; (4) Duplicate content of the included guidelines, or have a lower score on the guideline quality assessment; (5) The guidelines do not meet the criteria for research.

2.2.3. Evaluation of guideline quality and integration of evidence

The quality of the included evidence-based guidelines was assessed by two members of the protocol construction team using the Clinical Guideline Research and Evaluation System (AGREE II). The assessment results were independently entered into Excel by the pair to calculate the standardized scores of each guideline in different domains and to determine the level of recommendation. Meanwhile, the quality assessment tool of the Australian JBI Centre for Evidence-Based Health Care (2017 version) ^[6] was used to assess the quality of the expert consensus and professional opinion literature. For entries that were in disagreement during the evaluation process, a 3rd team member was involved in the discussion to reach a consensus. To ensure the reliability of the assessment results, the scores of the two evaluators were tested for consistency and the degree of agreement was measured by calculating the intragroup correlation coefficient (ICC). During the intensive reading stage of the guidelines, the two team members extracted relevant evidence from them and merged duplicate entries to remove contradictory or low-quality content. Eventually, the strength of evidence was classified according to the JBI Evidence-Based Practice Centre's criteria for evidence grading and recommendation levels to form the first draft of an evidence-based practice protocol for perioperative nutritional care for esophageal cancer.

2.2.4. Conducting expert group meetings

In the course of the meeting, the Expert Panel considered the first draft of the program line by line and made adjustments to the recommendations based on the quality of evidence, feasibility, effectiveness, and clinical

application value. The secretary is responsible for taking detailed notes of the meeting and making audio recordings for archiving. If there is disagreement on an entry, members of the meeting are required to discuss it together until a consensus is reached before proceeding to the next item. After all entries are revised, the moderator reviews the final revision to ensure that all experts unanimously approve before confirming the final proposal.

3. Results

3.1. Results of the guideline search and quality evaluation

A total of 111 pieces of relevant literature were retrieved in this study, and after screening the titles and abstracts, 27 literatures remained after excluding studies with incompatible types of literature, irrelevant topics, old versions of guides, duplicated literature, and non-compliant languages. After further reading of the full text, 13 documents were excluded for topic incompatibility, unavailability of the original text, translated version of the guidelines, and type non-compliance, and finally, 14 guideline documents were included, of which, two were domestic guidelines and 12 were foreign guidelines. Among the included guidelines, six were evidence-based guidelines and eight were consensus guidelines. After quality assessment, two of the six evidence-based guidelines were recommended at grade A, and four were recommended at grade B. The ICC values of the two evaluators were 0.5 and 0.5, respectively. The ICC value for both assessors was 0.961, indicating high consistency. In addition, the quality assessment of the consensus guidelines all met the inclusion criteria, with an inter-assessor ICC value of 0.885, again with high agreement.

3.2. Evidence is summarized to form a first draft

Based on the summary and analysis of evidence-based and consensus guidelines, a total of 75 evidence and recommendations related to nutritional care were extracted, which were derived from six evidence-based guidelines, and 27 recommendations screened from eight consensus guidelines. Subsequently, the research team conducted a preliminary collation of the collected content, excluding three entries that were controversial about whether percutaneous endoscopic gastrostomy was recommended for nutritional support preoperatively, removing 10 entries with duplicated content, and deleting nine entries that could not be formed into a specific protocol due to a lack of operability. Eventually, the integrated 80 recommendations were classified according to the nursing process and time points, and divided into four stages: admission, preoperative, postoperative, and discharge, to construct the first draft of the nutritional care practice plan applicable to the perioperative period of esophageal cancer patients.

3.3. Expert validation of final drafts

In this expert panel meeting, an in-depth validation of the perioperative nutritional care practice protocol was conducted. It was calculated that the coefficient of expert judgment (Ca) was 0.950, the degree of familiarity (Cs) was 0.875, and the comprehensive authority coefficient reached 0.912, indicating that the expert group had high authority and the opinions provided had a strong reference value. During the meeting, the experts actively discussed and put forward 34 suggestions for revision, including four content additions, 15 entry deletions, seven entry mergers, three content adjustments, and five suggestions for optimizing the program structure. After thorough discussions and revisions, the perioperative nutritional care practice protocol for esophageal cancer patients containing 61 entries was finalized. Among them, 33 entries were assessed as Grade A recommendations,

and 28 entries were classified as Grade B recommendations.

4. Discussion

4.1. Clinical importance of developing a perioperative nutritional care program for esophageal cancer patients

Perioperative nutritional management of esophageal cancer patients is crucial for surgical outcome and postoperative recovery, however, there is a lack of systematic and standardized guidance on the implementation of perioperative nutritional care in clinical practice, which leads to a large discrepancy in the implementation of nutritional interventions, and even affects the recovery process of patients. Therefore, it is of great clinical significance to construct a scientific and standardized perioperative nutritional nursing practice program^[7]. First of all, the program can provide an evidence-based basis for healthcare personnel to implement nutritional care more standardized and precise, to optimize the perioperative nutritional management process, and improve the quality of care. Defining the nutritional intervention strategies for each stage of admission, preoperative, postoperative, and discharge can help healthcare professionals assess the nutritional status of patients promptly, adopt individualized intervention measures, and reduce perioperative infections, poor wound healing, and delayed postoperative recovery caused by malnutrition. Secondly, through systematic nutritional care guidance, the program enables patients and their families to understand more clearly the importance of nutritional support and to cooperate with healthcare personnel in making reasonable dietary adjustments to ensure adequate preoperative nutritional reserves and postoperative promotion of recovery, which ultimately improves the prognosis. In addition, through the continuous optimization and improvement of the program, the further development of nutritional care for esophageal cancer can be promoted, the standardization and normalization of clinical care can be facilitated, and the overall quality of patient survival can be improved^[8]. This study focused on the perioperative period, the most significant stage of stress and trauma in esophageal cancer patients, and developed a scientific evidence-based practice program for nutritional care. The protocol covers four stages of care, namely admission, preoperative, postoperative, and discharge, and contains a total of 61 entries, aiming to provide a systematic and standardized nursing practice reference for clinical caregivers, optimize perioperative nutritional management, improve the quality of care, and promote patient recovery.

4.2. High clinical applicability of the nutritional care practice program constructed in this study

In constructing the nutritional care practice program in this study, the current situation of medical and nursing resources, cultural background, and baseline characteristics of patients in China were fully considered to ensure that the program had good clinical applicability. During the development of the program, an expert panel meeting was used to critically review the recommendations in the preliminary draft, focusing on assessing their clinical feasibility and practical application value.

For example, in the assessment of nutritional needs at the admission stage, the preliminary draft recommended the use of indirect calorimetry to measure the patient's resting energy expenditure. However, based on the clinical practice in China, the expert group pointed out that equipment such as metabolic carts required for indirect calorimetry is expensive and complicated to operate, and its popularity in domestic healthcare institutions is relatively low, so it is recommended that the simpler Harris-Benedict formula^[9]

be used for individualized estimation to improve the operability of the assessment method and the clinical diffusion of the method. For example, for the management of preoperative parenteral nutrition, the preliminary draft suggested that “when parenteral nutrition is administered via a peripheral vein, the osmolality should be controlled within 900 mOsm/L to ensure safety.” After discussion, the expert group concluded that the osmolality of parenteral nutrition solution is complicated to calculate, while plasma osmolality or saline osmolality is usually about 300 mOsm/L ^[10]. Therefore, to simplify the operation and to ensure that clinical caregivers can accurately grasp the standard, the panel recommends that the entry be amended to read, “Parenteral nutritional solutions entered through a peripheral vein are safe up to three times the tension.” Through the refinement of the applicability of each entry in the program, it will be more in line with the actual needs of China’s medical care environment, thus enhancing the feasibility and promotion value of the program in clinical practice.

5. Conclusion

In conclusion, this study summarized the best evidence of perioperative nutritional care for esophageal cancer through an evidence-based approach and finally formulated an evidence-based practice protocol for perioperative nutritional care for esophageal cancer patients through the deliberation and argumentation of an expert panel. The protocol not only demonstrates scientific rigor but also emphasizes clinical applicability. It provides practical guidance for nursing care, has high reference value, and lays a solid foundation for future promotion and application in clinical settings.

Disclosure statement

The authors declare no conflict of interest.

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Advances and Challenges in mRNA Vaccine Technology

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Abstract: Since the outbreak of COVID-19, mRNA vaccine technology has achieved groundbreaking advancements. Characterized by its high safety profile and potent immune activation capabilities, this technology has demonstrated significant potential in the prevention of infectious diseases and cancer therapeutics, marking a new milestone in vaccine development. This review focuses on three key aspects—molecular design, delivery systems, and immunological mechanisms—providing a comprehensive analysis of structural optimization strategies, delivery methodologies, and immune modulation approaches for mRNA vaccines. Additionally, it summarizes and evaluates potential challenges that may arise in the future development of mRNA vaccines. By analyzing existing technological pathways, this review aims to advance innovation in mRNA vaccine technology and facilitate its broad applications in public health and veterinary medicine.

Keywords: mRNA vaccine; Molecular design; Delivery systems; Immune mechanisms

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1. Introduction

Vaccines, as core biological agents in modern infectious disease prevention and control systems, have undergone iterative breakthroughs in development technologies such as inactivated, attenuated, recombinant protein, and gene-editing platforms. Through precise immune response mechanisms, these advancements have not only consigned virulent infectious diseases like smallpox to history but also continuously rewritten humanity's offensive and defensive strategies against pathogens. The novel coronavirus pneumonia (COVID-19) pandemic that broke out at the end of 2019 posed unprecedented challenges to global public health and underscored the urgency of vaccine development ^[1].

Unlike traditional vaccines, mRNA vaccines do not contain live pathogens or proteins. Instead, they utilize delivery systems to transport pathogen genetic information into host cells, providing instructions for intracellular processing and production of virus-related antigenic proteins, thereby activating the body's immune response ^[2].

Building on these unique modular design advantages and rapid response mechanisms, mRNA vaccine technology has broken through traditional R&D paradigms. Its distinctive antigen-coding flexibility and controllable immunogenicity have significantly improved cross-protective efficacy against viral variants, marking the entry of vaccinology into a new stage of precise regulation.

During the COVID-19 pandemic, Pfizer-BioNTech and Moderna successively announced clinical trial results for their COVID-19 mRNA vaccines. Data from phase 3 clinical trials showed that both vaccines achieved protective efficacy rates exceeding 94%, with a low incidence of severe adverse events. They were approved for marketing and use in the United States in December 2020 ^[3]. The successful application of mRNA vaccines has not only provided a powerful tool for pandemic control but also pointed out new directions for the development of vaccines against other infectious diseases. This article will review the molecular design, delivery systems, immune mechanisms, and challenges of mRNA vaccines.

2. Classification and molecular design of mRNA vaccines

mRNA vaccines are classified into non-replicating mRNA (NRM) vaccines, self-amplifying mRNA (SAM) vaccines, and circular RNA (circRNA) vaccines based on their genetic characteristics (**Figure 1**).

2.1. Non-replicating mRNA vaccine

NRM vaccines are currently the most widely used mRNA vaccine type in clinical applications. Their design is based on the structural framework of natural mRNA, including the 5' cap, untranslated region (UTR), open reading frame (ORF), and poly(A) tail. The optimized combination of these structural elements significantly enhances the stability, translation efficiency, and immunogenicity of mRNA.

2.1.1. 5' cap

The 5' cap is critical for mRNA stability and translation initiation. Common cap structures include cap 0 (m7GpppNp), cap 1 (m7GpppNmp), and cap 2 (m7GpppNmpNmp) ^[4]. Among these, cap 1, featuring 2'-O-methylation modification, effectively avoids recognition by the host innate immune system and has become the preferred choice for mainstream vaccines ^[4]. Capping methods are divided into enzymatic capping and co-transcriptional capping: enzymatic capping involves multi-step enzymatic reactions, while co-transcriptional capping uses cap analogs to directly generate cap 1 structures during *in vitro* transcription. Moderna's mRNA-1273 employs enzymatic capping, whereas Pfizer's BNT162b1 achieves high-efficiency capping via co-transcriptional methods ^[5,6].

2.1.2. Untranslated regions

The UTR optimization is another core strategy to improve mRNA performance. The 5' UTR enhances ribosome binding efficiency through the introduction of Kozak sequences, while the 3' UTR prolongs mRNA half-life by removing AU-rich degradation elements ^[7,8]. Additionally, UTR sequences from naturally highly expressed genes such as human hemoglobin α/β subunits (HBA/HBB), albumin (ALB), or heat shock proteins (Hsp70) can be used as direct substitutes ^[9].

2.1.3. Open reading frame

The ORF is the target antigen-coding region. Selecting appropriate optimization strategies in this region can enhance overall mRNA translation efficiency. Replacing uridine with chemically modified nucleotides such as pseudouridine or N1-methylpseudouridine significantly reduces mRNA immunogenicity while increasing resistance to RNases^[10]. Furthermore, rare codons in the ORF can be replaced based on the degenerate codon preferences of different hosts to improve translation efficiency^[11].

2.1.4. Polyadenylate tail

The poly(A) tail synergizes with the 5' end-cap structure to inhibit the degradation and stability of mRNA by exonucleases. The optimal poly(A) tail length varies by cell type, with studies suggesting a range of 120–150 nucleotides^[12,13]. BioNTech's segmented poly(A) tail design (A30LA70), incorporating a UGC linker, further extends mRNA retention time within cells^[14].

2.2. Self-amplifying mRNA vaccine

SAM vaccines are structurally similar to NRM vaccines but additionally encode viral RNA replication machinery-related genes, enabling self-amplification in host cells to induce high-level antigen expression at extremely low doses.

The core advantage of SAM vaccines lies in their “self-adjuvant effect.” After entering the cytoplasm, the SAM-encoded replicase is first translated and assembled into a multi-enzyme complex, which replicates the input mRNA into negative-strand RNA and subsequently generates new genomic mRNA and subgenomic mRNA. The latter drives efficient antigen protein expression via subgenomic promoters while activating strong innate immune responses (i.e., the “self-adjuvant effect”). For example, the LNP-nCoVsaRNA vaccine developed by Imperial College London, based on Venezuelan equine encephalitis virus (VEEV) genes, requires only 0.1–10 µg to induce high-titer neutralizing antibodies in mice^[15].

However, the long sequences (typically exceeding 9 kb) and complex secondary structures of SAM vaccines pose production challenges. To address this, researchers have developed trans-amplifying RNA (taRNA) vaccines by separating the replicase gene and antigen gene into two distinct RNA molecules^[16,17]. This dual-vector system not only simplifies production but also minimizes replication-induced interference with host cells. For instance, in influenza vaccine studies, 0.05 µg taRNA achieved complete protection in mice, demonstrating ultra-high dose efficiency^[17].

2.3. Circular RNA vaccine

CircRNA vaccines are an emerging form of mRNA vaccines characterized by a covalently closed circular structure. Unlike linear mRNA, circRNA resists exonuclease degradation without requiring a 5' cap or poly(A) tail, enabling prolonged intracellular persistence. Its closed structure also evades recognition by pattern recognition receptors, reducing innate immune responses and dependence on nucleotide modifications^[18]. However, circRNA translation efficiency is limited by the activity of internal ribosome entry sites (IRES). Studies show that traditional virus-derived IRES can drive translation but with far lower efficiency compared to the cap-dependent mechanism of linear mRNA^[19]. Additionally, the design of long circRNA sequences and residual linear RNA contaminants during production complicate manufacturing processes.

To overcome these challenges, circRNA vaccine optimization focuses on enhancing translation efficiency

and practicality. Research has identified IRES from human rhinovirus B (HRV-B), enterovirus B (EV-B) (e.g., iHRV-B3 and iEV-B107) that exhibit stronger translational activity in circRNA. Inserting synthetic aptamers to enhance interactions with translation initiation factors can significantly boost translation efficiency ^[19]. Currently, circRNA vaccine development remains in its early stages.

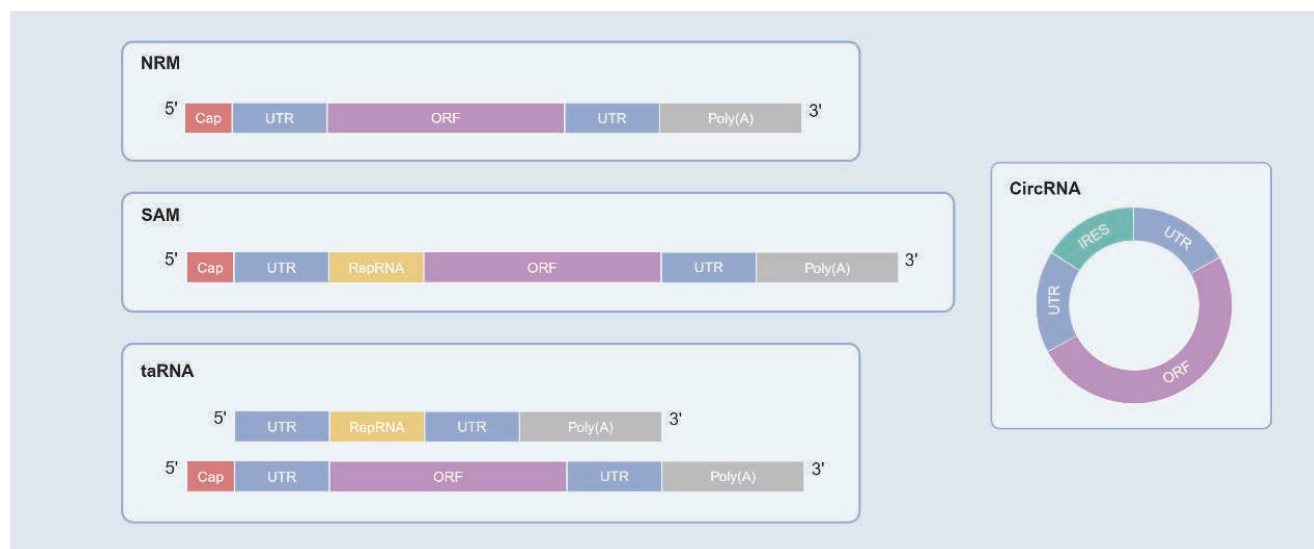


Figure 1. Molecular design of different types of mRNA vaccines

3. Delivery systems for mRNA vaccines

mRNA must cross the cell membrane to enter the cytoplasm to exert its effects. However, due to its large molecular weight and negatively charged nature, mRNA cannot penetrate the cell membrane and is prone to recognition and degradation by the immune system. Therefore, it is very necessary to develop efficient mRNA vaccine delivery systems. To date, scientists have achieved significant progress in mRNA vaccine delivery systems, which can be broadly classified into two categories: physical delivery and carrier-assisted delivery methods (**Figure 2**).

3.1. Physical delivery of naked mRNA

Physical delivery methods overcome the cell membrane barrier through external forces to directly introduce exogenous mRNA into target cells or tissues. These primarily include direct injection, electroporation, and gene gun technologies.

3.1.1. Direct injection

Direct injection of mRNA is commonly used in cancer therapy, with major administration routes including intramuscular, subcutaneous, intradermal, and intralymphatic approaches. In 2013, Phua *et al.* ^[20] found that subcutaneous injection of naked mRNA in mice achieved higher delivery efficiency than mRNA nanoparticle delivery methods. Van Lint *et al.* ^[21] proposed that intertumoral injection of tumor-associated mRNA triggers appropriate immune responses and could become a promising vaccination strategy. Recently, more and more researchers have focused on the role of naked mRNA in treating or preventing infectious diseases. The team

of Abbasi ^[22] employed a needle-free jet injector (PYRO) that utilizes transient liquid pressure to promote the internalization of naked mRNA into skin cells. This method induced local lymphocyte infiltration, significantly reduced viral load in challenged mice, alleviated tissue damage, and provided effective immune protection.

3.1.2. Electroporation

Electroporation is one of the most widely used physical delivery techniques. Its principle involves applying a brief high-voltage electric field to create transient pores in the cell membrane, facilitating mRNA entry into cells. Since its first application in gene transfection in 1982, electroporation has been commonly used for *in vitro* mRNA transfection of hematopoietic cells ^[23]. In tumor immunotherapy, electroporation is employed for dendritic cell (DC) mRNA transfection, activating T-cell immune responses through tumor antigen-encoding mRNA. For example, clinical trials in melanoma patients demonstrated that electroporation-delivered mRNA induces robust CD4⁺/CD8⁺ T-cell responses ^[24]. Additionally, electroporation exhibits adjuvant effects by recruiting pro-inflammatory cells and inducing cytokine secretion, thereby enhancing mRNA immunogenicity ^[25]. However, limitations include potential cell membrane damage or apoptosis, and its superior immune-enhancing effects in SAM (self-amplifying mRNA) over NRM (non-replicating mRNA) *in vivo* applications restrict its broader use ^[26].

3.1.3. Gene gun technology

Gene gun technology uses compressed gas (helium or nitrogen) to propel gold-coated mRNA particles at high speed into target tissues, achieving delivery through physical penetration. Studies have confirmed that gene guns can deliver human α -1 antitrypsin mRNA to mouse skin and elicit antibody responses ^[27]. Subsequent developments applied this technology to mRNA repair therapies for genetic skin diseases, successfully targeting deeper skin layers in mice ^[28]. Although highly efficient in murine models, its efficacy in large animals and humans remains unverified. Moreover, the physical impact of gold particles may disrupt normal cellular physiology or cause local tissue damage, limiting its clinical translation potential.

In general, the core advantage of physical delivery technology is to bypass vector dependence and directly realize the intracellular delivery of mRNA. In addition, some methods (e.g., electroporation) also function as immune adjuvants. However, its drawbacks should not be ignored: on the one hand, physical external forces may cause cell damage or death, affecting the safety of treatment; On the other hand, technologies such as gene guns are inefficient in the transformation of large animals and humans, and it is difficult to meet clinical needs. In addition, the positioning accuracy of physical methods on target tissues is limited, and it is difficult to achieve systemic delivery. These limitations have prompted research to move to safer, controlled delivery systems such as lipid or polymer nanoparticles. Nevertheless, physical delivery still has irreplaceable value in specific scenarios, such as local tumor therapy or skin targeting, and its efficacy and safety need to be further improved through technological improvements in the future.

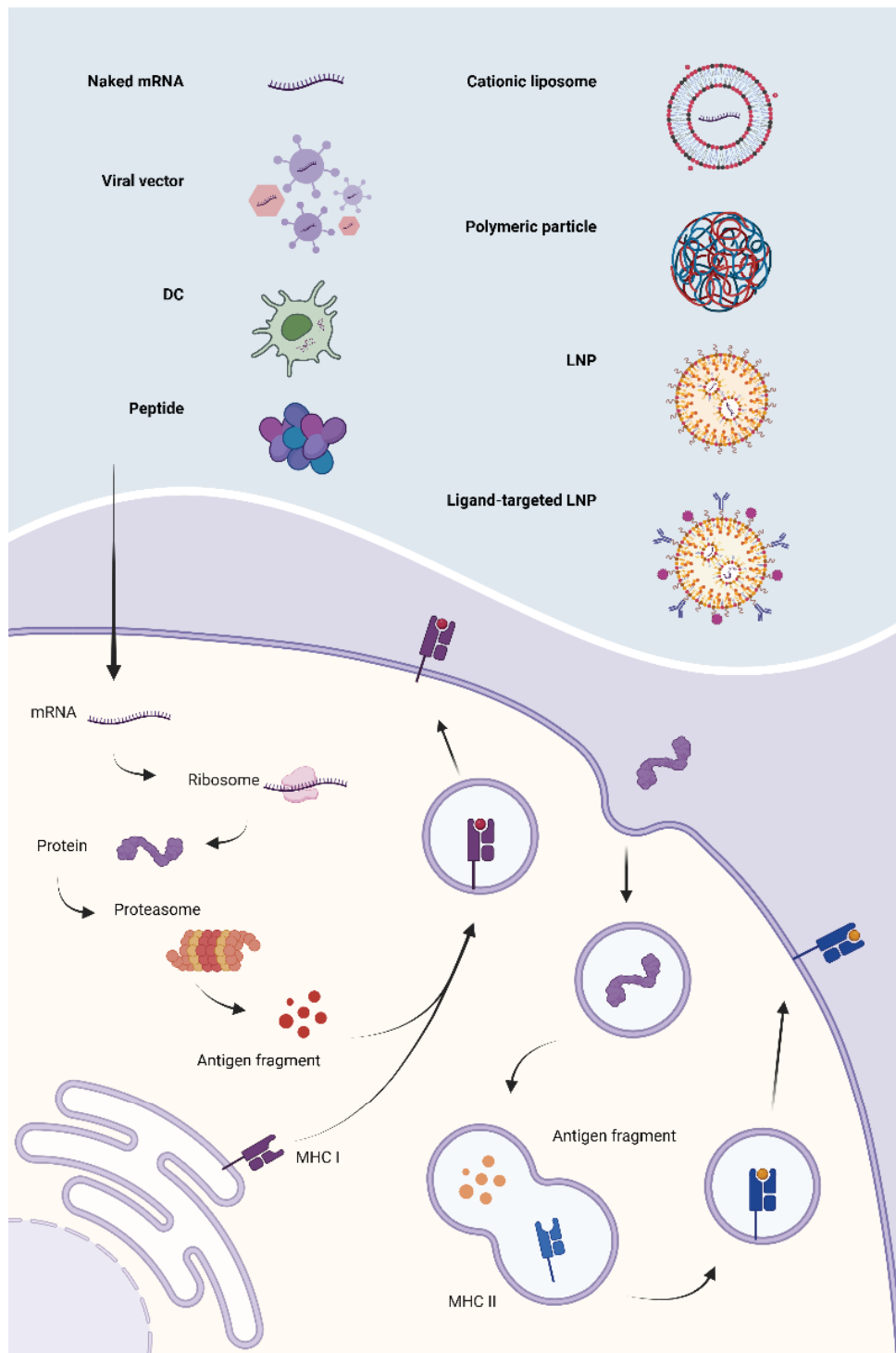


Figure 2. mRNA vaccine delivery mechanisms and adaptive immune response mechanisms

3.2. Carrier-based mRNA delivery methods

Despite the direct intracellular delivery provided by physical methods, their inherent limitations have driven researchers toward more biocompatible carrier systems. Compared to physical interventions, carrier delivery employs biomimetic or engineered designs to mimic natural cellular interaction mechanisms, enhancing mRNA stability and enabling precise delivery. Current carrier systems are divided into biological and non-biological

categories: the former utilizes viral or cellular bioactive units, while the latter relies on synthetic nanomaterials. Below, we systematically elaborate on carrier-based mRNA vaccine delivery technologies and their clinical potential, focusing on breakthroughs in immune activation efficiency, tissue specificity, and safety.

3.2.1. Biological carriers

3.2.1.1. Viral vector

Viral vectors have long been used for RNA drug delivery. Retroviral vectors, among the earliest developed, remain the preferred choice for *ex vivo* transfection of hematopoietic stem cells. Adeno-associated virus (AAV) vectors are widely used in *in vivo* studies due to their safety, broad host cell range, and prolonged transgene expression, making them one of the most common gene therapy vectors^[29]. Lentiviral vectors can infect both dividing and non-dividing cells, accommodate inserts up to 8 kb, and enable long-term expression^[30]. Studies show that viral vector-delivered mRNA formulations are effective against viral and bacterial diseases and cancer^[31]. However, risks such as genomic integration, uncontrollable gene expression, and severe immune side effects limit their clinical adoption.

3.2.1.2. Dendritic cell

DCs are the most potent antigen-presenting cells (APC). After capturing antigens in tissues, they migrate to lymphoid organs to present processed antigens to immune cells, initiating cellular and humoral immunity. The DC-mRNA delivery approach involves transfecting DCs *ex vivo* with antigen-encoding mRNA and reinfusing them into the host to activate specific immune responses. DC-mRNA systems are favored in clinical research for their high delivery efficiency without additional carriers and their strong induction of cellular immunity, particularly in cancer therapy^[32]. However, complex and costly production processes hinder large-scale application. Additionally, immune responses triggered during *ex vivo* mRNA transfection may diminish during preparation, reducing therapeutic efficacy^[33]. These issues necessitate further optimization for clinical use.

3.2.2. Non-biological carriers

3.2.2.1. Cationic lipids

Cationic lipids were the first-generation lipids developed for mRNA vaccine delivery. Their inherent positive charge enables spontaneous complexation with negatively charged mRNA and attraction to cell membranes. N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium (DOTMA) and its synthetic analog 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) were among the first cationic lipids used for mRNA delivery. Lipofectin, a cationic liposome formulation developed from these, demonstrated strong cell transfection capabilities^[34].

However, the cationic nature may lead to nonspecific interactions with negatively charged serum proteins, forming aggregates that shorten half-life and cause adverse effects^[35]. To address this, second-generation ionizable lipids were developed. Structurally optimized with tertiary amine groups, these lipids remain neutral at physiological pH but protonate to become positively charged in acidic environments. This pH sensitivity allows these lipids to better circumvent strong interactions with cell membranes during circulation *in vivo*, which in turn significantly reduces their immunogenicity and biotoxicity and improves delivery efficiency^[36].

3.2.2.2. Polymers

Polymers can be divided into cationic and anionic polymers, and positively charged cationic polymers are widely

used for mRNA delivery. Polyethylenimine (PEI) is the most researched and commercialized cationic polymer in the field of mRNA delivery, consisting of linear or branched polymers with different relative molecular weights and structures^[37]. Due to its intrinsic cationic properties, PEI binds tightly to mRNA through electrostatic interactions, efficiently delivering it into the cell, and releasing mRNA within the cytoplasm through the cleavage of disulfide bonds. However, high positive charge density induces cytotoxicity^[38]. To address this challenge, researchers have found that the addition of chemical modifications can significantly improve the delivery and biological tolerance of PEI *in vivo*^[39]. In addition, a variety of amine-containing polyester polymers have been developed for mRNA delivery. Polyester-based polymers can significantly improve biodegradability and biocompatibility by introducing labile chemical bonds, including carbonate, ester, amide, and phosphate bonds^[40].

3.2.2.3. Peptides

Due to electrostatic action, negatively charged mRNA is easily adsorbed by positively charged peptides for delivery. Protamine is a small molecular weight protein rich in arginine that binds tightly to mRNA to form a stable complex, protects mRNA from RNase degradation in serum, and acts as an adjuvant to elicit a strong immune response^[41]. In recent years, protamine delivery platforms have been developed for clinical applications in various vaccines and cancers, showing great prospects for development^[42,43].

Cation cell-penetrating peptide (CPP) is another small molecule peptide with excellent delivery ability, low charge density, and cell membrane disrupting properties, which can effectively enhance the endosomal escape efficiency of mRNA^[44]. The commercial peptide PepFect14 has been shown to be effective in delivering therapeutic mRNA to ovarian cancer cells in mouse models^[45]. Although CPP has shown great potential in the field of vaccine delivery, the US Food and Drug Administration (FDA) has not approved any CPP-conjugated compounds for clinical use due to their lack of tissue specificity and cytotoxicity^[46].

3.2.2.4. Lipid nanoparticles

Lipid nanoparticles (LNPs) are currently the most advanced and widely used mRNA delivery system and are the only drug delivery system that has been clinically proven effective and approved for human use^[47]. LNPs are nanoscale vesicles capable of mimicking the lipid structure of cell membranes to encapsulate mRNA in their cavities. LNPs are composed of four components: ionizable lipids, pegylated lipids (PEGs), cholesterol, and auxiliary phospholipids. The self-positive charge of ionizable lipids facilitates the self-encapsulation of mRNA and facilitates the escape of mRNA from endosomes upon entry into cells. Hydrophilic PEG can improve the colloidal stability of LNP in biological fluids and prolong the half-life of LNP. Cholesterol and phospholipids primarily support the structural stability of LNP particles^[48].

Previous studies have shown that LNPs can greatly promote mRNA delivery *in vivo* and enhance antigen expression, resulting in durable protective immune responses against a variety of infectious agents^[49,50]. Companies such as Moderna, Pfizer, and BioNTech have used LNPs in the development of COVID-19 mRNA vaccines against the novel coronavirus (SARS-CoV-2). Many other studies have also validated the efficacy of LNP-mRNA in cancer therapy^[51,52]. Although LNP-mRNA vaccines have the advantages of good biosafety, mature industrial production technology, and convenient large-scale production, there are still problems in clinical application, such as low cell uptake rate and prone to inflammatory reactions. Studies have shown that the delivery efficiency of LNPs can be improved by adjusting or modifying the lipid components of liposomes and selecting the appropriate route of administration^[53].

3.2.2.5. Targeted delivery

LNPs bind to apolipoprotein E (APOE) and target APOE receptors on the surface of hepatocytes, resulting in most systemically administered LNP-mRNAs targeting only the liver^[54]. Therefore, the specific delivery of LNP-mRNA to specific organs and cells has become a major technical challenge. Targeted delivery technology enables precise delivery of mRNA to target cells and tissues by integrating antibodies or aptamers in nanoparticles^[55]. This method uses the high-affinity interaction between antigen-antibody or aptamer-receptor to improve the specificity and efficiency of mRNA delivery and reduce the possibility of causing excessive inflammation, which is currently a research hotspot in the field of mRNA drug delivery.

4. Immune mechanisms of mRNA vaccines

The immune mechanism of mRNA vaccines involves the activation of innate and adaptive immunity, as well as the dynamic presentation process of antigens *in vivo*. At its core, antigenic proteins are synthesized using the host cell's translation system and elicit specific protective responses against pathogens through multi-level immune signaling.

4.1. Activation and regulation of innate immunity

Innate immunity is the body's first line of defense against non-self-infused substances, and after exogenous mRNA enters the host cell, pathogen-related molecular patterns in its molecular structure can be recognized by a variety of pattern recognition receptors. For example, single-stranded RNA (ssRNA) is recognized by Toll-like receptors in endosomes (eg, TLR7, TLR8), while double-stranded RNA (dsRNA) contaminants are recognized by RIG-I-like receptors (eg, RIG-I, MDA5) in the cytoplasm or TLR3 in endosomes^[56]. The binding of these receptors triggers a downstream signaling cascade that ultimately promotes the secretion of type I interferons and pro-inflammatory cytokines.

Type I interferon has a dual role in immune activation: on the one hand, it lays the foundation for the initiation of adaptive immunity by promoting the maturation of DCs and macrophages, enhancing antigen presentation ability; On the other hand, excess interferon inhibits the translation mechanism of host cells through the JAK-STAT signaling pathway, resulting in reduced antigen expression efficiency of mRNA vaccines^[57]. Therefore, balancing the degree of activation of innate immunity is key to optimizing mRNA vaccines.

In order to reduce the negative impact of excessive immune activation on vaccine efficacy, nucleotide modification and novel purification techniques are widely used as feasible methods to improve the efficacy of mRNA vaccines^[58,59]. The modified mRNA can significantly reduce the recognition ability of TLR7 and TLR8, reduce the secretion of type I interferon, and improve the stability and translation efficiency of mRNA. Purification processes such as high-performance liquid chromatography (HPLC) can remove dsRNA contaminants from *in vitro* transcribed mRNA, which can avoid abnormal activation of RIG-I and MDA5 and further balance immune stimulation and antigen expression.

4.2. Initiation and amplification of adaptive immunity

After being delivered to the host cell by a vector, the mRNA vaccine escapes from the endosomal to the cytoplasm and is translated by the ribosome into the target antigen protein. A part of the antigenic protein is degraded into

short peptides by the proteasome in the cytosol, which binds to MHC class I molecules and presents them to the cell surface, activating CD8⁺ T cells and initiating cellular immunity. The other part is secreted extracellular, engulfed by APC as an exogenous antigen, and after being degraded by lysosomes, it binds to MHC class II molecules to activate CD4⁺ T cells and initiate humoral immunity (**Figure 2**).

Notably, mRNA vaccines have the unique advantage of being able to directly transfect antigen-presenting cells, thereby achieving cross-presentation. For example, DCs can present antigens to CD8⁺ T cells via the MHC class I pathway after ingestion and translation of mRNA, while activating CD4⁺ T cells via the MHC class II pathway. This dual-pathway activation mechanism significantly enhances the synergistic effect of T cells, which not only enhances the breadth and potency of cellular immunity but also provides long-lasting and effective humoral immune protection by maintaining germinal center responses to form long-lived plasma cells and memory B cells ^[60,61].

5. Current challenges for mRNA vaccines

5.1. Insufficient durability of antibody responses

Whether it can induce durable immune protection after vaccination is one of the core indicators to measure its effectiveness. After mRNA vaccination, the antigen produced is captured by the APC and transferred to the lymph nodes, promoting the formation of germinal centers. In this process, B cells, APCs, and follicular helper T cells (Tfh cells) work together to drive the production of high-affinity neutralizing antibodies ^[62]. Although preclinical studies ^[63,64] have shown that mRNA vaccines can induce strong germinal center responses and Tfh cell activation against a variety of pathogens such as human immunodeficiency virus (HIV), Zika virus (ZIKV), SARS-CoV-2, etc., the duration of antibody responses varies significantly depending on antigenic properties. As an example, the Pfizer vaccine BNT162b2 detected a strong germinal center B cell response for at least 12 weeks after vaccination, while the Moderna vaccine mRNA-1273 retained high and high antibody levels for six months, but antibody titers gradually decreased over time ^[65,66]. In addition, virus mutations may lead to a weakening of antibody-neutralizing efficacy. Studies have shown that cross-neutralizing antibody titers against the B.1.351 and P.1 variants of the novel coronavirus are significantly lower than those of the original strains ^[67,68]. Therefore, the development and design of mRNA vaccines targeting conserved epitopes may be a key strategy to prolong immune protection.

5.2. Need for safety optimization

Although existing mRNA vaccines have shown good safety in clinical trials and practical applications, there is still a need for further optimization. Dose-dependent adverse effects are a major concern, such as the fact that Moderna's H10N8 influenza vaccine experienced serious adverse events in the 400 microgram dose group, which ultimately led to the adjustment of the maximum dose to 100 micrograms ^[69]. In addition, the incidence of anaphylaxis with mRNA vaccines was significantly higher than with conventional vaccines, with anaphylaxis occurring at 2.2 and 2.5 per million with the Pfizer-BioNTech and Moderna vaccines, respectively ^[70]. Researchers hypothesize that these allergic reactions are related to PEG components: anti-PEG antibodies are present in about 40% of the population through daily toiletries, which may induce IgM production and accelerate allergic reactions through T-cell-independent pathways that activate B-cell receptors ^[71]. In preclinical studies, the presence of anti-PEG IgM in animal serum accelerates the clearance of nanoparticles and leads to a complete loss of efficacy for mRNA therapeutics ^[71,72]. It can be seen that improving the formulation of vaccine ingredients and reducing PEG

dependence will become the future improvement direction of mRNA vaccines.

5.3. Limitations in accessibility and stability

The requirement for cold-chain storage of mRNA vaccines has severely constrained their rollout in low- and middle-income countries. The Pfizer-BioNTech vaccine needs to be stored at ultra-low temperatures at -70°C , while the Moderna vaccine also needs to be at least -20°C , posing a huge challenge for areas with weak infrastructure. In order to prevent the recurrence of a global pandemic, it is necessary to develop a plan for heat-stabilized dosage forms. Preclinical studies have shown that some mRNA vaccines can be stored at room temperature using sequence optimization or lyophilization^[73,74]. If these technologies are validated in clinical trials, they will significantly reduce transportation and storage costs and expand vaccine coverage. In addition, increasing production capacity and reducing production costs are also keys to improving accessibility, especially in the face of new variants that require rapid iteration of vaccines, and the flexibility and scale of production will determine the efficiency of response.

5.4. Unique challenges in veterinary applications

In the field of animal medicine, the promotion of mRNA vaccines faces a series of unique challenges, which to a certain extent delay the industrialization process of this technology in the prevention and control of livestock diseases.

First, the core challenge in vaccine development stems from the deep heterogeneity of immune systems between species. Studies have shown significant species differences in mammalian Toll-like receptor signaling pathways, distribution of antigen-presenting cell subsets, and cytokine regulatory networks^[75,76]. As a result, mRNA vaccines developed based on the same pathogen may exhibit very different immune response characteristics in different species. In addition, the choice of adjuvant needs to be carefully weighed: polyinosidylate can effectively enhance Th1 immunity in porcine models, but may induce an excessive inflammatory response in felines; Although aluminium adjuvant can increase avian antibody titers, it may inhibit the cellular immune response in rodents^[77-80]. Therefore, it is important to establish a design framework for species fitness based on systematic vaccinology. Researchers need to integrate multi-dimensional parameters such as epitope prediction of B/T cells of target animals, species-specific codon optimization algorithms, and biocompatibility assessment of delivery systems to achieve accurate cross-species mRNA vaccine development.

Second, the contradiction between economy and large-scale production is particularly prominent. The livestock industry needs low-cost, high-coverage vaccines to prevent and control large-scale outbreaks, and while mRNA technology has the advantage of rapid adaptation to new pathogens, the high standards of plasmid purification and dsRNA contaminant removal in GMP manufacturing can drive up costs. In addition, large-scale farmed animals often need to be vaccinated by non-injection methods such as oral administration and spraying, which puts forward higher requirements for the stability and delivery efficiency of mRNA. Preclinical studies have shown that mRNA encapsulated in LNPs is easily degraded in a simulated gastric acid environment, and how to develop a delivery system that can tolerate the digestive tract environment is an urgent problem to be solved^[81,82].

In addition, the duality of regulatory and safety standards also makes the development of mRNA vaccines more difficult. Animal vaccine approvals are often more cost-effective than safe, but consumers may be skeptical about the residual risk of mRNA vaccines in food animals. Although available data suggest that mRNA components can be rapidly degraded in animals, further research is needed on whether lipid carriers (e.g., PEGs)

can be delivered to humans through meat or dairy products^[26,83]. Regulators need to establish a cross-species safety assessment framework and promote public education to dispel misconceptions such as “genetically modified animal products.”

6. Conclusion

Through molecular design optimization, delivery system innovation, and immune mechanism analysis, mRNA vaccine technology has established its milestone position in the prevention and control of infectious diseases and tumor treatment. The pseudouridine modification and segmented poly(A) tail design of NRM significantly improved the stability, and the low-dose and high-efficiency expression characteristics of SAM provided a new idea for broad-spectrum immunity, while the closed structure of circRNA broke through the restriction of enzyme degradation, but still needed to solve the bottleneck of translation efficiency. In the delivery system, LNP has become the mainstream due to its targeting and industrial maturity, but the problems of PEG-related allergic reactions and hepatic enrichment still need to be broken through. The study of immune mechanisms revealed the core advantages of mRNA vaccines in activating dual-pathway adaptive immunity through cross-presentation, but the challenges of insufficient antibody persistence, viral escape mutations, and cold chain dependence still restrict their application and promotion.

In the future, the development of mRNA vaccines needs to focus on three directions: first, deepen molecular design, develop targeted conserved epitope and species-appropriate sequences, and improve clinical practicability. Second, innovate delivery technology, develop reliable carriers that are resistant to digestion or stable at room temperature, and explore de-PEGylated lipids and organ-specific targeting strategies to reduce the risk of immunogenicity. Third, promote process upgrading, optimize production costs and accessibility through technological innovation, and establish a cross-species safety assessment framework to accelerate the industrialization of veterinary vaccines. At the same time, expanding application scenarios to cancer, gene editing, and rare disease treatment will be the key to technological breakthroughs.

The continuous evolution of mRNA technology will not only reshape the emergency response system for emerging infectious diseases but also lead to a new era of personalized medicine and precision immunotherapy. Through interdisciplinary collaboration to overcome the barriers to stability, safety, and transformation, it is expected to achieve full coverage from human public health to animal health management, providing a solution with both speed and effectiveness to the global health crisis.

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Research Progress of EZH2 in Tumors and Translational Perspectives

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Abstract: Enhancer of zeste homolog 2 (EZH2) is a key epigenetic regulatory protein and enzyme catalytic subunit of the polycomb repressor complex 2 (PRC2), responsible for catalyzing the trimethylation of histone H3K27 and subsequent repression of gene transcription. Abnormal *EZH2* expression or mutation is associated with various cancers, particularly lymphoma, and breast and prostate cancer. EZH2 has been investigated as an important target in cancer therapy and potential EZH2-targeted drugs have been developed. This article reviews the research progress on the mechanism of transcriptional regulation of *EZH2* and the development and clinical use of some inhibitors targeting EZH2.

Keywords: EZH2; Polycomb repressor complex 2; Cell signaling; Inhibitor

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1. Introduction

The *EZH2* gene is located on human chromosome 7q36.1. It has 20 exons and 19 introns and encodes 746 amino acid residues. Each of the 10 EZH2 protein domains has a distinct structure and function. These domains include the SANT1L-binding domain (SBD), the EED-binding domain (EBD), the β -addition motif (BAM), the SET-activation loop (SAL), the stimulus-responsive motif (SRM), the motif that connects SANT1L and SANT2L (MCSS), two SANT structural domains, the CXC domains and SET domains (**Figure 1A**)^[1]. The histone methyltransferase activity of EZH2 is mainly maintained by the SET structural domain, and the CXC structural domain is also required for binding to the SET structural domain. The structural domain at the N-terminal end of the SET structural domain is a protein interaction domain, that is required for the assembly of the corresponding subunits for polycomb repressor complex 2 (PRC2) function^[2]. PRC2 is a member of the PCG proteins, an evolutionarily conserved class of protein complexes originally discovered in *Drosophila*^[3]. PRCs consist primarily of two core complexes: polycomb repression complex 1 (PRC1) and polycomb repression complex 2 (PRC2)^[4]. The PRC2 complex is an S-adenosyl-L-methionine (SAM)-dependent histone methyltransferase (HMTases), which contains four major core subunits: EZH2/1, ZESTE12 (SUZ12), embryonic ectodermal developmental

2.2. PRC2-dependent non-histone methylation

Beyond its traditional role in histone methylation, EZH2 has also been found to methylate non-histone proteins. One notable target is GATA4, a process that is dependent on PRC2. This methylation at lysine residues of GATA4 by EZH2 directly hinders its transcriptional function. Such type of repression plays a crucial epigenetic regulatory role in processes of development and cell differentiation through non-histone methylation ^[11].

2.3. PRC2-independent gene transactivation

In addition to its traditional gene silencing function, EZH2 also has a transcriptional activation function that helps tumor cells survive and escape the effects of DNA damage by activating specific genes ^[12]. EZH2 can function as a coactivator to directly activate androgen receptor (AR) transcriptional activity. Therefore, it can play a non-catalytic role as it is PRC2 and methylation-independent ^[13].

3. Dysregulation of EZH2 and related signaling pathways in tumors

As one of PRC2's main constituents, EZH2 is often dysregulated. As shown in **Figure 1**, its role in tumors is often accompanied by genetic changes. In most cases, mutations and amplification, as well as overexpression, provide the driving force in the process.

3.1. Dysregulation of EZH2

One of the most common alterations affecting epigenetic mechanisms is the gain-of-function mutations in *EZH2*. Studies have shown that seven different activating mutations affect the catalytic SET structural domain of the protein, with mutations located in exon 16 and exon 18 ^[14]. Activating mutations in *EZH2*, such as the common mutation at the Y646 site, improve its ability to catalyze H3K27 trimethylation. This leads to abnormal silencing of tumor suppressor genes, which in turn promotes abnormal cell proliferation and tumor formation ^[15]. This is more common in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) ^[16]. In DLBCL and FL, gain-of-function mutations were also identified in residues Y641, A677, or A687 in the catalytic domain of *EZH2* ^[17,18].

Studies have shown that somatic mutations in the *EZH2* gene are also present in myelodysplastic syndromes (MDS) and that these mutations, which can impair or completely lose the methyltransferase activity of EZH2, are associated with a poor prognosis and can also accelerate the progression of MDS to acute myeloid leukemia (AML) ^[19]. Stasik *et al.* revealed a low frequency but significant adverse prognostic impact of *EZH2* mutations in AML by analyzing genomic data from 1,604 newly diagnosed AML patients, a finding that provides potential direction for personalized treatment of AML, particularly the development of targeted therapies against EZH2 ^[20].

The PBRM1-SWI/SNF complex and EZH2 exhibit an antagonistic relationship in epigenetic and cell cycle regulation. In renal cell carcinoma (RCC) with *PBRM1* mutations, EZH2 activity is frequently upregulated, potentially driving accelerated cancer cell proliferation. Consequently, EZH2 inhibitors are anticipated to represent a promising targeted therapeutic approach for *PBRM1*-mutant RCC ^[21]. Overexpression of EZH2 has been found in a variety of solid tumors, including lung cancer ^[22], breast cancer ^[23], melanoma ^[24], prostate cancer ^[25], and bladder cancer ^[26].

3.2. EZH2 activates the PI3K/AKT/mTOR signaling pathway

The PI3K/AKT signaling pathway has a well-established effect on the initiation and spread of cancer. In cancers, this signaling is essential for fostering cell division and viability ^[27]. Studies have shown that AKT hyperphosphorylates Rb and releases large amounts of E2F1, which activates cell cycle proteins and cell cycle protein-dependent kinases (CDKs). When E2F1 binds to the EZH2 promoter, transcription of *EZH2* is activated ^[28].

EZH2 can activate the PI3K/AKT/mTOR signaling pathway by epigenetically silencing the expression of PTEN via increasing the H3K27me3 level. PTEN is an important negative regulator that inhibits this signaling pathway ^[29,30]. PTEN and PTENP1 expression are suppressed when YY1 and EZH2 cooperate, which raises the phosphorylation level of S473-AKT and T308-AKT and activates AKT ^[31]. Thus, EZH2 forms a positive feedback loop with AKT. Moreover, EZH2 can also directly promote PI3K and AKT signaling pathways by interaction with the PI3K or AKT signaling components.

Studies have shown that interaction between KDM2B and EZH2 results in increased levels of AKT and PI3K in colorectal cancer ^[32]. EZH2 increases AKT phosphorylation by activating TNFSF13B ^[33].

3.3. EZH2 activates the Wnt/ β -catenin signaling pathway

The Wnt/ β -catenin signaling pathway plays a variety of roles, such as cell proliferation, differentiation, and migration. Down-regulation of Wnt significantly reduces the level of β -catenin and cyclin D1 ^[34]. There are two mechanisms of action: (1) EZH2 promotes sustained Wnt signaling activation by silencing Wnt signaling repressors such as DKK1 and SFRP1. Through histone H3K27me3 modification, EZH2 reduces the expression of these repressors, which in turn deregulates the negative feedback regulation of Wnt signaling, therefore β -catenin can accumulate and translocate to the nucleus, where it activates the expression of downstream oncogenes ^[35,36]. (2) Studies have shown that EZH2 can directly interact with β -catenin to stabilize it in the nucleus and increase its transcriptional activity. This interaction contributes to the binding of β -catenin to Tcf/Lef and promotes the expression of Wnt target genes, thereby promoting tumor cell proliferation and invasion ^[37].

3.4. EZH2 and other signaling pathways

In cervical cancer HeLa cells, it has been reported that EZH2 binds to the CARD structural domain of VISA and disrupts the interaction between RIG-I and VISA, thereby attenuating downstream signaling and the innate immune response ^[38]. GAS5 induces apoptosis of smooth muscle cells and subsequent abdominal aortic aneurysms via activation RIG-I signaling pathway mediated by EZH2 ^[39]. PVT1 is a long-stranded non-coding RNA that interacts with EZH2 in non-small cell lung cancer (NSCLC), mediates methylation of the miR-497 promoter, and prevents the upregulation of miR-497 and YAP1 ^[40].

EZH2-mediated upregulation of microRNA-375 promotes breast cancer progression by inhibiting FOXO1 and p53 signaling pathways ^[41]. Downregulation of EZH2 activates the JNK signaling pathway and increases the expression levels of inflammatory cytokines such as TNF- α , IL-17, IL-5, CCL20, and CCL2 ^[42]. EZH2 can promote bladder cancer proliferation and migration via the JAK2/STAT2 pathway ^[43].

3.5. EZH2 is involved in the metabolic reprogramming

Metabolic reprogramming is a process whereby tumor cells change their metabolic pathways to accommodate the demands of rapid growth and spread. Epigenetic control plays an important role in cancer development ^[44]. A crucial metabolic characteristic of the Warburg phenotype, aerobic glycolysis, is caused by active metabolic

reprogramming, which is necessary for the long-term growth and malignant development of cancer cells ^[45].

3.5.1. EZH2 and lipid metabolism

Disturbance of lipid metabolism is a striking metabolic change in cancer. Lipid metabolism is used for energy production, as components of biofilms, and as signaling molecules for proliferation, survival, invasion, metastasis, and tumor microenvironmental response in cancer cells ^[46]. EZH2 is a major regulator of lipid metabolism. Cumulative evidence suggests that EZH2 methyltransferase activity, which is required for adipogenesis during preadipocytes and adipogenesis, promotes differentiation of the adipocyte by epigenetic repression of the genes of the Wnt signaling pathway ^[47].

In cancer cells, dysregulation of lipid metabolism reduces the sensitivity to the EZH2-specific inhibitor GSK126, and treatment with GSK126 results in increased synthesis of lipids, as evidenced by increased unsaturated fatty acids ^[48]. GSK126 also induced lipid accumulation in human adipocytes without altering the expression of marker genes for adipocyte differentiation, one mechanism may be that inhibition or knockdown of EZH2 promoted lipoprotein-dependent lipid uptake and increased apolipoprotein E (ApoE) expression. Studies have shown that the absence of ApoE prevented GSK126 from promoting lipid-independent lipid accumulation in mouse adipocytes ^[49].

In an animal model, EZH2-deficient mice were leaner than normal mice and had less white adipose tissue in their bodies. Compared to controls, EZH2-deficient mice had smaller lipid droplets in brown adipocytes and more beige adipocytes (a type of cell that is intermediate between white and brown fat). Meanwhile, mice in the *EZH2* knockout group had reduced differentiation markers in white adipocytes and increased UCP1 and other browning markers in brown and beige adipocytes, better tolerance to cold stimuli, and resistance to obesity and insulin resistance induced by a high-fat diet ^[50]. Histone deacetylase 1 (HDAC1) negatively regulates the thermogenic program of brown adipocytes through the synergistic effect of EZH2-mediated H3K27 deacetylation and methylation. These findings provide new insights into the role of epigenetic regulation in metabolic regulation and could provide a basis for the development of new therapies to treat metabolic diseases such as obesity ^[51].

3.5.2. EZH2 and glucose metabolism

Even with sufficient oxygen supply, cancer cells obtain energy primarily through glycolysis, the so-called Warburg effect. In prostate cancer, EZH2 regulates aerobic glycolysis and cell growth by acting on the miR-181b/hexokinase 2 (HK2) axis ^[52]. However, the opposite effect is observed in that EZH2 inhibits tumor cell proliferation by inhibiting GLS expression and reducing glutamine metabolism, which is distinct from its classical role as the central histone methyltransferase of the PRC2 complex ^[53].

SIRT3 plays an important role in glycolysis and proliferation in colorectal cancer cells ^[54]. EZH2 inhibits the expression of SIRT3 by directly binding to its promoter region and decreases SIRT3 levels, resulting in increased sensitivity of radioresistant cells to glucose starvation. EZH2 inhibitors can increase the tolerance of cancer cells to glucose starvation by eliminating this inhibitory effect. Therefore, modulating SIRT3 expression by targeting EZH2 may help overcome radioresistance and improve therapeutic response to nutrient deficiency ^[55].

It has also been shown that activation of the EZH2/FBXL7/PFKFB4 axis resulted in the hypoxic environment allowing tumor cells to better adapt to glucose-deficient conditions and increase energy supply, which in turn accelerated NSCLC progression ^[56].

4. Advances in studying EZH2 as a therapeutic target

EZH2 plays a critical role in the pathophysiology of cancer and is therefore a potential target for cancer therapy. Melanoma is a malignant skin tumor, and EZH2 is considered a potential therapeutic target for melanoma as it plays a role in the development and progression of melanoma^[57]. Silencing of EZH2 with siRNA or treatment with DZNep (or MS1943) inhibited the growth of hypochromic melanoma cells and induced hyperchromic melanoma cells^[58]. Epigenetic silencing of interferon gene-stimulating factor (STING) mediated by EZH2 resulted in low STING expression in melanoma cells. Targeted therapies consisting of EZH2 inhibitors (EZH2i) and STING agonists have improved preclinical antitumor immunity in melanoma^[59]. EZH2i can not only directly inhibit CRC cell proliferation, but also regulate macrophages by tilting M2 macrophages toward effector M1 macrophages, thus exerting an anti-tumor effect^[60].

High EZH2 expression in ovarian cancer is closely associated with a high tumor proliferation index, advanced tumor stage, and poor prognosis. Human cytomegalovirus infection has been associated with ovarian cancer and plasmacytoid ovarian cancer biopsy tissues are characterized by increased expression of EZH2, but also results in polyploidization, which can be observed as polyploid giant cells of tumors with cancer stem cell-like characteristics^[61].

4.1. EZH2 inhibitors

EZH2 is a valuable target for cancer treatment and many EZH2 inhibitors have been extensively studied, including tazemetostat, GSK126, DZNPEP, and others.

Tazemetostat reduced β -catenin and CD13 protein expression in HepG-2 cells and their HBV-transfected cells through inhibition of EZH2, thereby reducing the survival of hepatocellular carcinoma cells. One study provides theoretical support for the potential use of tazemetostat in the treatment of hepatitis B-associated hepatocellular carcinoma and highlights the key role of EZH2 as a therapeutic target^[62]. It had also been shown that tazemetostat has an anti-cancer activity against tumor cells in esophageal cancer^[63]. The inhibitor is also a potential therapeutic option for overcoming multidrug resistance (MDR) in tumors and can synergize with a variety of conventional chemotherapeutic agents *in vitro*, especially in cancers resistant to conventional treatments, and has broad clinical applications^[64]. In a recent clinical trial, tazemetostat demonstrated some clinical efficacy and was well tolerated in pediatric tumors with *SMARCB1/SMARCA4* or *EZH2* genetic alterations^[65].

By using the EZH2 inhibitor DZNep, cell proliferation in germinal center B-cell-like diffuse large B-cell lymphoma (GCB-DLBCL) can be reduced. This may be mediated by upregulating p16. This suggests that DZNep could be a potential therapeutic option for GCB-DLBCL^[66]. GSK-126, another effective inhibitor of EZH2, can affect apoptosis and protect brain cells after cerebral ischemia^[67]. In a subcutaneous A375 xenograft mouse model, oral administration of ZLD1039 (100 mg per kg) selectively reduced H3K27 methylation in melanoma cells by inhibition of EZH2 methyltransferase activity^[68].

IHMT-337 is a novel irreversible EZH2 inhibitor that prevents malignant tumor cell proliferation by simultaneously inhibiting EZH2 activity and downregulating *CDK4* transcription. This discovery provides a new idea for the development of targeted anticancer drugs against EZH2 and demonstrates a significant antitumor effect of IHMT-337 in preclinical models^[69]. Another novel dual-target PARP1/EZH2 inhibitor, KWLX-12e, was used in wild BRCA-type triple-negative breast cancer and was nontoxic to normal breast cells. By inhibiting EZH2, KWLX-12e increases sensitivity to PARP1 lethality and induces cell death, representing a potential candidate for the treatment of triple-negative breast cancer^[70].

DYB-03 is also a dual-target drug of HIF-1 α and EZH2. Molecular predictions indicate that DYB-03 may form multiple hydrogen bonds with both proteins, effectively inhibiting their functions. The study also showed that DYB-03 has strong antitumor activity *in vitro* and *in vivo* (including in a mouse tumor model of transplantation) and was able to significantly inhibit lung cancer cell migration, invasion, and angiogenesis and promote apoptosis. In addition, DYB-03 may reverse the resistance of lung cancer to existing drugs such as 2-ME2 and GSK126^[71,72].

Huang and colleagues designed a dual EZH2-BRD4 inhibitor with excellent pharmacological activity by analyzing the nature of the heterodimeric compounds and the detailed structure-activity relationships. The compound demonstrated excellent inhibitory activity and cytotoxicity *in vitro* and was able to induce apoptosis and reduce tumor cell proliferation *in vivo*. These results suggest that the compound is a potential therapeutic agent for the treatment of solid tumors and provides a new idea for the development of novel EZH2-BRD4 dual inhibitors^[73]. The above-mentioned inhibitors are listed in **Figure 2**.

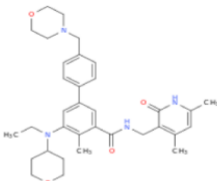
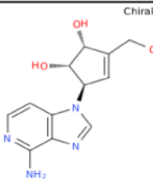
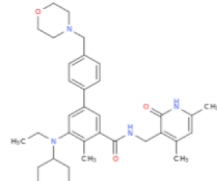
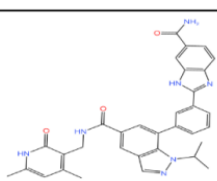
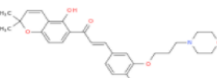
name	Compound structure	cancer type
Tazemetostat (EPZ6738)		liver cancer, pediatric tumor
DZNep (3-Deazaneplanocin A)		GCB-DLBCL
IHMT-337		lymphoma, prostate cancer, breast cancer
KWLX-12e		triple-negative breast cancer (TNBC)
DYB-03		lung cancer

Figure 2. Major inhibitors targeting EZH2

4.2. Combination therapy with EZH2 inhibitors

Advances in immunotherapy and targeted therapies have improved the treatment of tumors, but not all patients respond to these therapies and a large proportion of patients suffer from drug resistance. EZH2 is overexpressed in a variety of tumors and the mechanism of tumorigenesis has been extensively studied. In acquired or intrinsically

resistant ccRCC models, inhibition of EZH2 expression or activity restores the antitumor effect of sunitinib by inhibiting the phosphorylation of specific receptor tyrosine kinases. This suggests that EZH2 inhibitors have a better therapeutic effect when combined with other anticancer drugs ^[74].

By regulating the expression of immune-related genes, EZH2 helps tumor cells evade recognition by T cells and other immune cells, thereby promoting immune escape. The lncRNA EPIC1 is involved in many cellular processes that promote cell viability and invasion and cell cycle progression by interaction with MYC. Guo *et al.* showed that EZH2 is a key regulator in EPIC1-mediated tumor immune escape and immunotherapeutic resistance ^[75].

In breast cancer with BRCA1 deficiency, the combination of EZH2 inhibitors (e.g. tazemetostat) and ATM inhibitors significantly inhibits cell proliferation and induces DNA damage, resulting in cell death. This combination therapy has shown strong anti-tumor activity in *in vivo* and *in vitro* studies and provides a potential therapeutic strategy for treating BRCA1-defective cancers, particularly in patients resistant to existing therapies ^[76].

Androgen deprivation therapy (ADT) is a commonly used treatment for recurrent prostate cancer. After a period of response, almost all patients develop ADT resistance. BET and EZH2 inhibitors work better together than either one alone to effectively suppress cell viability, proliferation, and clonogenicity in metastatic prostate cancer cells ^[77].

When used in combination, BRAF inhibitors and EZH2 inhibitors significantly inhibit melanoma cell growth and increase apoptosis. This combination therapy is more effective than BRAF inhibitors or EZH2 inhibitors alone and has the potential to overcome drug resistance in particular ^[78].

5. Conclusion and prospects

EZH2 is a key histone methyltransferase and has been extensively investigated. The fact that EZH2 upregulation is closely related to the onset, progression, metastasis, and invasion of tumors is indicative of EZH2 being a prominent cancer target.

As a result, a number of specific inhibitors against EZH2 have been developed. Tazemetostat is the first EZH2 inhibitor available for clinical use. However, the genomic heterogeneity of tumors, complex epigenetic regulatory networks, and drug resistance may be reasons for the variable efficacy of EZH2 inhibitors. The development of dual-target drugs against EZH2 has led to new advances in optimizing tumor treatment. Moreover, a large body of evidence shows that chemotherapy drugs in combination with EZH2 inhibitors have a stronger antitumor effect. To improve the limitation of EZH2 inhibitors in clinical treatment, optimization of various combinations of metabolic modulators, immunotherapy, radiotherapy, and chemotherapy is a promising therapeutic strategy in the near future.

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Disclosure statement

The authors declare no conflict of interest.

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Antioxidant Protective Effect of Melatonin on Cyclophosphamide-Induced Premature Ovarian Failure and its Mechanism

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Abstract: *Objective:* To study the antioxidant protective effect and mechanism of melatonin on cyclophosphamide-induced premature ovarian failure model mice. *Methods:* Six-month sexually mature female Kunming mice were taken for one week of acclimatization and then randomly divided into a normal group, blank control group, drug control group, ovarian premature aging model group, and melatonin intervention low, medium, and high dose group, with 20 mice in each group. We observed the status and body mass of the mice in each group; observed and monitored the estrous cycle by HE staining; measured the diameter and size of the ovaries and weighed the wet weight of the ovaries; observed the morphological changes of the ovaries by HE staining and counted the developing follicles at all levels; detected the levels of serum estradiol (E2), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) by ELISA; measured the levels of serum MDA, SOD, and GSH-PX by antioxidant kit; detected the levels of protein immunoblotting by ELISA; protein immunoblotting (Western blot) to examine the expression of DNA damage-related proteins γ H2AX, p53, and p21 in ovarian tissues. *Results:* Compared with the control group, mice in the premature ovarian failure model group showed reduced mobility, rough hair, decreased body weight, disorganized estrous cycle, decreased ovarian weight ($P < 0.05$), decreased number of follicles at all levels of development ($P < 0.05$), increased number of atretic follicles ($P < 0.05$), significantly elevated levels of serum FSH and LH, significantly decreased levels of E2 ($P < 0.05$), significantly increased levels of serum MDA, significantly lower SOD and GSH-PX levels ($P < 0.05$), and the expression of p53, p21, and γ H2AX in ovarian tissues was increased ($P < 0.05$). Compared with the model group of premature ovarian failure, melatonin improved the changes of the above indexes induced by cyclophosphamide-induced premature ovarian failure in mice. *Conclusion:* Melatonin can improve the changes of motility cycle disorders, abnormal follicular development, and abnormal serum hormone levels induced by cyclophosphamide-induced oxidative stress in mice with premature ovarian failure. At the same time, melatonin can improve the oxidative stress induced by cyclophosphamide and alleviate the role of oxidative stress-induced DNA damage in mouse ovaries by exerting its antioxidant effect.

Keywords: Melatonin; Cyclophosphamide; Premature ovarian failure; Antioxidation

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1. Introduction

The global incidence of premature ovarian failure (POF) is approximately 1–7%. In China, the incidence of this disease accounts for about 2.8%, and there is an increasing trend year by year and younger patients being affected ^[1,2]. POF refers to the amenorrhea phenomenon before the age of 40 caused by the exhaustion of female ovarian function. Clinical manifestations include high follicle-stimulating hormone levels (FSH > 40 U/L with testing periods of over one month), declined estrogen levels (LH < 73.2 pmol/L), perimenopausal symptoms such as strange menstruation (scanty or amenorrhea), hot flashes and anguish, sorrowful mood, concern dozing, doubled incidence of heart disease, and dropped bone mineral density, which are the causes of abnormal reproductive health in women ^[3]. The occurrence of POF is related to genetics, radiotherapy and chemotherapy treatment, ovarian surgery history, autoimmune dysfunction, and metabolic disorders ^[4-6]. Among them, chemotherapy-induced POF has become a clinical concern ^[7]. Cyclophosphamide (CTX), a commonly used chemotherapeutic drug for anti-tumor treatment in clinical practice, belongs to the alkylating agent category. It has the greatest toxic effect on the female reproductive system, especially the ovaries, and exhibits a dose-dependent effect. CTX can promote ovarian granulosa cell death, resulting in an aberrant reduction in the number of ovarian follicles, depletion of ovarian follicular reserves, ovarian dysfunction, and eventually premature ovarian failure ^[8-10]. Melatonin (MT), an indoleamine hormone secreted by the mammalian hypothalamic pineal gland, is an often utilized free-radical scavenger in clinical practice. It is secreted in a circadian rhythm, reaching a peak at night. According to research, it has been suggested that the concentration of MT in human follicular fluid is higher than that in the blood ^[11]. Numerous previous studies have confirmed that MT can improve ovarian oxidative stress through its antioxidant capacity, thereby inhibiting ovarian granulosa cell apoptosis, preserving ovarian reserve function, improving fertility, and enhancing pregnancy outcomes ^[12-14]. This study intends to explore the antioxidant protective effect and molecular mechanism of MT on CTX-induced premature ovarian failure by using MT on a CTX-induced premature ovarian failure mouse model. It is hoped that this will provide a basic theoretical foundation and new ideas for clinical preventive treatment of chemotherapy-induced premature ovarian failure.

2. Materials and methods

2.1. Materials

2.1.1. Experimental animals and environment

A total of 140 sexually mature female Kunming mice at 6 weeks of age, weighing 28–30 g, with normal estrus cycles, were purchased from the Lanzhou Veterinary Research Institute (License: Lanzhou Veterinary Research Institute SCXk2020-0002). Housing conditions: room temperature 20–26°C, indoor humidity 55–70%.

2.1.2. Drugs and reagents

Melatonin powder and cyclophosphamide powder were purchased from Shanghai Macklin Biochemical Technology Co., Ltd., with catalog numbers M813985-25g and C849559-500mg, respectively; 4% paraformaldehyde; hematoxylin and eosin (H&E) staining solution from Feijing Biotechnology Co., Ltd.; malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) detection kits from Nanjing Jiancheng Bioengineering Institute; estradiol (E2), FSH, LH ELISA kits from Shanghai Enzyme-linked Biotechnology Co., Ltd.; protein extraction reagent and BCA protein concentration determination kit from Xin Sai Mei Biotechnology Co., Ltd.; p21 rabbit monoclonal antibody from Abcam; γ H2AX mouse monoclonal antibody

(catalog number: JBW301) from Shanghai Baili Biotechnology Co., Ltd.; p53 rabbit polyclonal antibody and GAPDH rabbit antibody from Jiangsu Qinke Biotechnology Research Center Co., Ltd.; horseradish peroxidase-labeled goat anti-rabbit and goat anti-mouse secondary antibodies from Hangzhou Hua'an Biotechnology Co., Ltd.; 5× protein loading buffer (containing DTT) from Solaibo Company; universal antibody diluent from Suzhou Xin Sai Mei Biotechnology Co., Ltd.

2.1.3. Main instruments

The instruments included multi-functional full-wavelength microplate reader from Meigu Molecular Instruments (Shanghai) Co., Ltd.; Amersham Imager 600 electrophoresis imaging analysis system from GE Healthcare Bio-Sciences AB; incubator from Jintan Tianjing Experimental Instrument Factory; HWS-12 electric constant temperature water bath from Zhejiang Nade Scientific Instrument Co., Ltd.; German sigma high-speed frozen centrifuge 3k15 from Shanghai Lingyi Biotechnology Co., Ltd.; AX224ZH electronic balance from Ohaus Instrument Co., Ltd.; small electrophoresis instrument from Xi'an Tengling Biotechnology Co., Ltd.

2.2. Experimental methods

2.2.1. Animal grouping, modeling, and drug administration

Adaptive feeding for 5-week-old female Kunming mice for one week was followed by selecting mice with stable estrus cycles for subsequent experiments. The mice were randomly assigned to seven groups, with 20 mice per group: Normal group (Group A): No intervention; Blank control group (Group B): Intraperitoneal injection and gavage of normal saline; MT administration group (Group C): MT (15 mg/kg) gavage; CTX-induced POF model group (Group D): First-day intraperitoneal injection of 50 mg/kg CTX + continuous 14-day intraperitoneal injection of 8 mg/kg CTX ^[15]; Synchronous combined protection group: Low-dose Group E: Group D CTX dose + MT (7.5 mg/kg) gavage, Medium-dose Group F: Group D CTX dose + MT (15 mg/kg) gavage, High-dose Group G: Group D CTX dose + MT (30 mg/kg) gavage; MT was administered by gavage every day at 20:00 for 14 consecutive days ^[16].

2.2.2. Growth status and body mass changes

Following the modeling, mice in each group were observed and recorded during drug administration and feeding in order to observe their feeding, fur, and activity. At the same time, the body mass changes of the mice were monitored every other day and recorded, and the drug dosage was adjusted according to the body mass.

2.2.3. Changes in the estrus cycle

Every morning at 8 am, vaginal exfoliated cell detection was performed on mice in each group. The specific operation method is as follows: The mouse was held steady with one hand, while a pipette was held with the other to draw 200 µL of normal saline solution. The pipette was gently inserted into the mouse's vagina, and the saline solution was injected, followed by repeated rinsing 2 to 3 times. The rinsed solution was then aspirated and spread onto a pre-numbered slide. After settling and fixation, H&E staining was performed. The estrous cycle was determined using an optical microscope, recorded, and represented in a line chart. The normal estrus cycle of mice is stable for 4 to 5 days. When there is a prolongation of diestrus, continuous diestrus, or the estrus cycle of the mice becomes chaotic and lasts for more than 6 days, or there is a long-term stagnation in a certain period, it is considered that the mice have estrus cycle disorders and ovarian damage ^[17]. Microscopic observation

showing dominant flaky anuclear keratinized epithelial cells indicates estrus; when nuclear oval epithelial cells are dominant, it is proestrus; when a large number of white blood cells are dominant, it is diestrus; when all three types of cells exist, it is metestrus ^[18].

2.2.4. Changes in ovarian size and wet weight

After the modeling was completed, the mouse's whiskers were cut off, blood was taken from the eyeballs, and then the mouse was euthanized. The ovarian tissue was taken out, fat tissue was removed, and the ovarian length and wet weight were measured and recorded.

2.2.5. Changes in the pathological morphology of ovarian tissue

After soaking the ovarian tissue in 4% paraformaldehyde solution, it was inserted into paraffin, sectioned, and stained with H&E. The ovarian tissue morphology was observed under a light microscope, and the number of follicles at each stage was counted. The ratio of atretic follicles to total follicles (proportion of atretic follicles) was calculated. Microscopic follicle evaluation: Primordial follicles are defined as oocytes surrounded by a layer of squamous granulosa cells; primary follicles have oocytes surrounded by a layer of cubic granulosa cells; secondary follicles are surrounded by more than one layer of cubic granulosa cells, and follicular cavity has not yet formed; early antral follicles begin to develop a follicular cavity, and preovulatory antral follicles show distinct layered granulosa cell layers; atretic follicles have oocytes that are shrinking and irregularly shaped, with deeply stained zona pellucida ^[19].

2.2.6. Detection of serum hormones

Blood was collected from the eyeball and placed in a 1.5 ml EP tube, permitted to stratify at 4°C in a refrigerator, and then centrifuged at 3500r/min for 10 minutes at 4°C in a low-temperature centrifuge. The ELISA method was utilized to detect serum levels of FSH, E2, and LH, strictly adhering to the guidelines provided by the ELISA kit. The remaining serum was stored in a -80°C freezer for future use.

2.2.7. Measurement of serum MDA, SOD, and GSH-PX

Frozen serum samples were thawed and used according to the antioxidant kit's instructions to measure the levels of MDA, SOD, and GSH-PX in mouse serum.

2.2.8. Western blot detection of related pathway protein expression

Ovaries stored at -80°C were removed and crushed, and proteins were extracted. After detecting protein concentration using a BCA kit, 5× loading buffer was added according to the volume, boiled in water for 10 minutes, and then stored at -20°C for future use. Equal amounts of protein were electrophoresed on a 6% or 10% SDS polyacrylamide gel. The gel was run at 80V until it exited the stacking gel (approximately 30 minutes), then at 100V until the end of the resolving gel (approximately 2 hours). A 5.5 cm × 8 cm PVDF membrane was cut, and a sandwich layer was made in the order of the black board, sponge pad, filter paper, gel, PVDF membrane, filter paper, sponge pad, and white board. The membrane was transferred at 120 mA for 2.5 hours. After transfer, the membrane was washed three times with 1× TBST for 10 minutes each, and then blocked with 5% BSA for one hour. The membrane was cut and incubated with primary antibodies p53 (1:1000), p21 (1:800), γH2AX (1:1000), and GAPDH (1:4000) at 4°C overnight. Following the conclusion of the incubation phase, the membrane was

subjected to three washes with $1\times$ TBST, each wash lasting for a period of 10 minutes. Subsequent to the removal of the primary antibody, the membrane was placed into a chamber containing HRP-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies, where it remained for a duration of one hour at ambient temperature. Protein blots were visualized with enhanced luminescent reagent, and protein gray values were analyzed using ImageJ software. The expression levels of other proteins were calculated using GAPDH as an internal reference.

2.2.9. Statistical methods

The measured data obtained was statistically analyzed using Graph Pad Prism 10.1 software. Each experiment was repeated three times. Multiple groups were compared using one-way ANOVA, and further comparisons between two groups were performed using a *t*-test. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of MT on the general condition of CTX-induced POF mice

Mice in the normal group and the blank control group had smooth hair, a good mental state, normal activity, and normal weight gain. Compared with the control group, the model group had sparse hair, rough and dull skin, significantly reduced activity, and lower body weight. Mice in the MT-only administration group and the low, medium, and high MT dose groups exhibited shiny hair and varying degrees of weight gain compared to the model group; however, these differences were not statistically significant (see **Figure 1**).

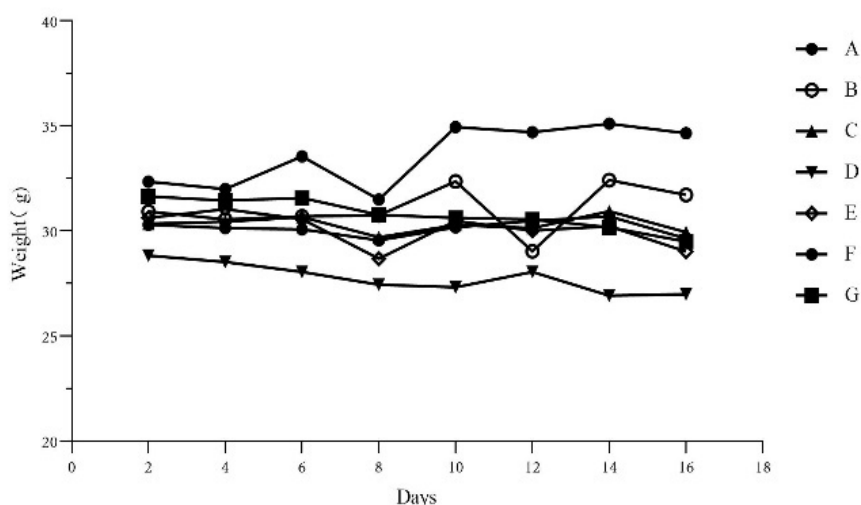


Figure 1. Line graph of changes in body weight of mice in each group. Note: Group A: Normal group, Group B: Control group, Group C: MT only group, Group D: Premature ovarian failure model group, Group E: Low-dose MT group, Group F: Medium-dose MT group, Group G: High-dose MT group.

3.2. The effect of MT on the estrus cycle of CTX-induced POF mice

The H&E staining results of vaginal exfoliated cells from mice are illustrated in **Figure 2**. After modeling, the estrus cycles of mice in the normal group, blank control group, and MT-only administration group were normal, averaging 4–5 days per cycle. However, in comparison to the blank control group, the premature ovarian failure model group exhibited disordered estrus cycles and varying degrees of estrus cycle prolongation, averaging 7–9

days per cycle, and some mice did not show a complete estrus cycle. After MT protection was administered, the estrus cycles of mice in each group showed varying degrees of recovery, with estrus cycles ranging from 5–7 days per cycle, which was shorter and more stable compared to the premature ovarian failure model group.

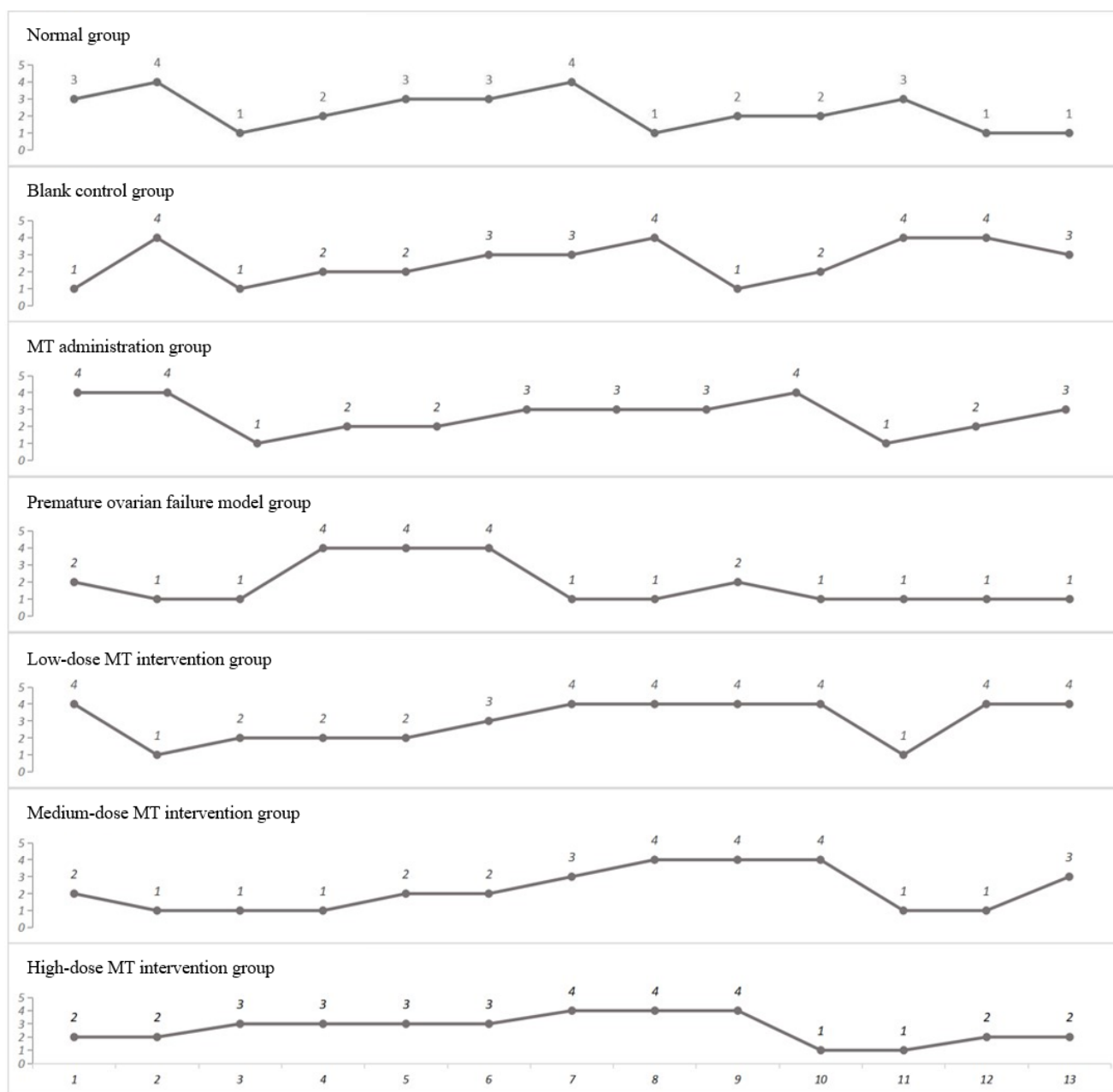


Figure 2. Line graph of the motility cycle during treatment in mice. Note: 1: Proestrus, 2: Estrus, 3: Metestrus, 4: Diestrus.

3.3. The effect of MT on ovary size and wet weight in CTX-induced POF mice

In the absence of statistical differences between the normal group and the blank control group, compared to the blank group, the ovarian diameter of mice in the model group was slightly reduced, but the difference was not statistically significant ($P > 0.05$). However, the ovarian wet weight of mice in the model group and the MT low, medium, and high dose groups were significantly reduced ($P < 0.01$, $P < 0.05$). There were no statistically significant changes in ovarian diameter and wet weight in the MT-only administration group ($P > 0.05$). Compared

to the model group, the ovary size and wet weight of mice in the MT low, medium, and high dose groups showed varying degrees of increase, but the difference was not statistically significant ($P > 0.05$), as shown in **Figure 3**.

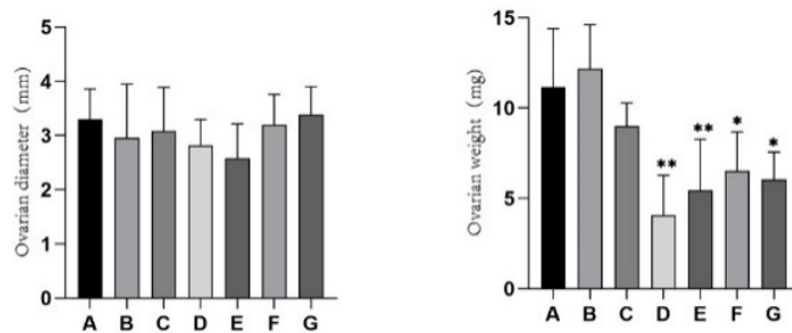


Figure 3. Comparison of ovary diameter and wet weight in each group of mice. Note: Compared with the control group: $P < 0.05$, $*P < 0.01$; compared with the premature ovarian failure model group: $\#P < 0.05$, $\##P < 0.01$. Group A: Normal group, Group B: Control group, Group C: MT-only group, Group D: Premature ovarian failure model group, Group E: Low-dose MT group, Group F: Medium-dose MT group, Group G: High-dose MT group.

3.4. The effect of MT on the pathological morphology of ovarian tissue in CTX-induced POF mice

Statistical analysis of ovarian histopathological sections and follicle counts at various stages is shown in the figures. A large number of follicles and corpora lutea at various stages can be seen in the ovaries of mice in the normal group, control group, and MT-only administration group, with rare atretic follicles (see **Figure 4**). Compared to the control group, the number of primordial follicles, preantral follicles, and mature follicles in the model group mice was significantly reduced ($P < 0.05$, $P < 0.01$), while the number of atretic follicles was significantly increased compared to the control group ($P < 0.005$), with statistically significant differences. Compared to the model group, the number of primordial follicles, preantral follicles, and mature follicles in the ovaries of mice in the medium and high-dose MT treatment groups was significantly increased ($P < 0.05$, $P < 0.01$), and the number of atretic follicles was significantly reduced ($P < 0.005$, $P < 0.01$). However, compared to the control group, the changes in primordial follicles, preantral follicles, and mature follicles in the low-dose group were not significant, with only a noticeable reduction in the number of atretic follicles ($P < 0.05$).

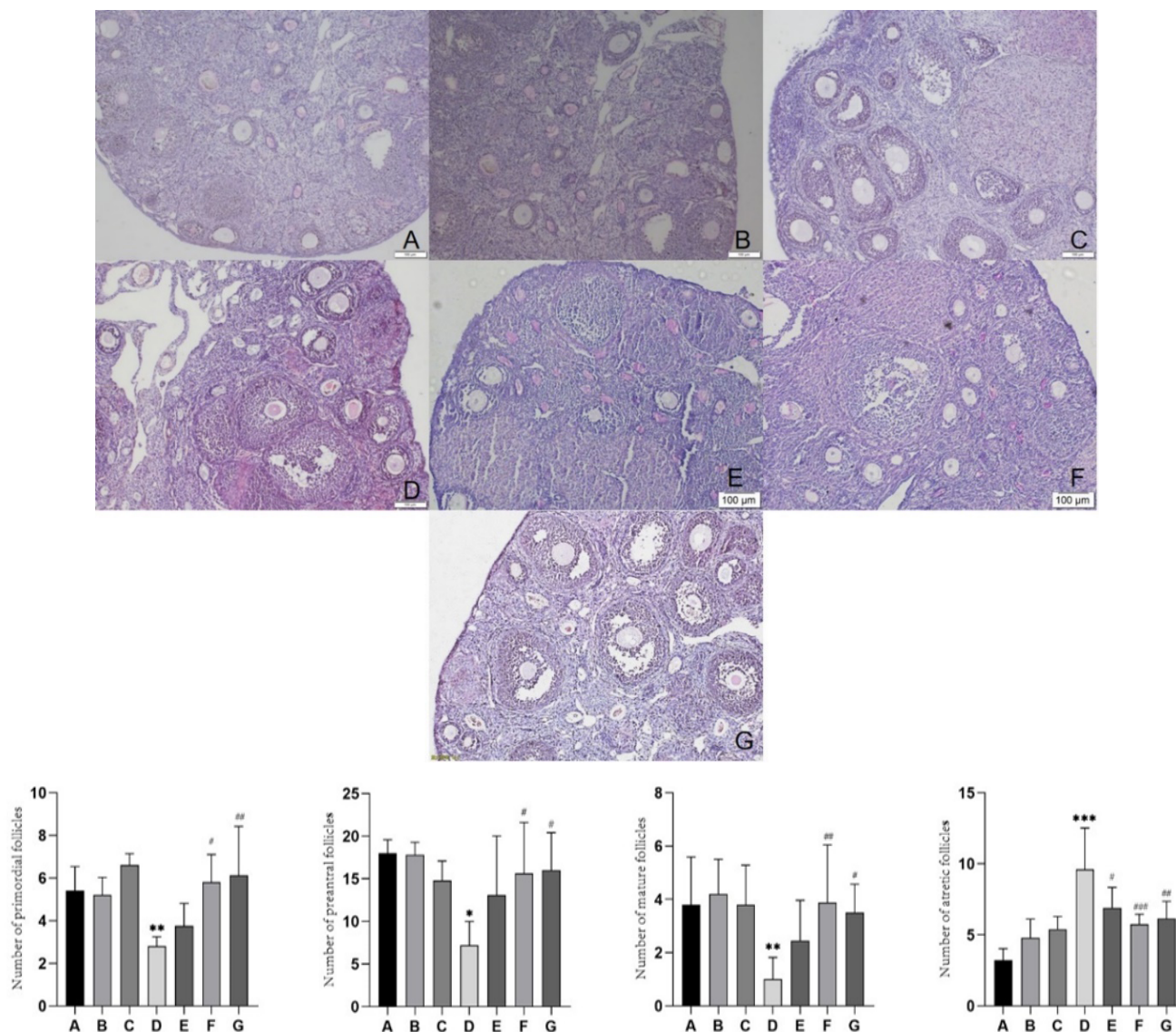


Figure 4. Morphological changes in ovarian tissue (H&E $\times 100$) and follicle counts at all levels in all groups of mice. Note: Compared with the control group: $P < 0.05$, $P < 0.01$, $**P < 0.005$; compared with the premature ovarian failure model group: $\#P < 0.05$, $\##P < 0.01$, $###P < 0.005$. Group A: Normal group, Group B: Control group, Group C: MT-only group, Group D: Premature ovarian failure model group, Group E: Low-dose MT group, Group F: Medium-dose MT group, Group G: High-dose MT group.

3.5. The effect of MT on serum hormones in CTX-induced POF mice

The levels of E2, FSH, and LH in the serum of mice in each group are shown in **Figure 5**. There were no statistically significant differences in the three hormone levels between the normal group and the blank control group ($P > 0.05$). Compared to the control group, the levels of LH and FSH in the serum of the model group were significantly increased ($P < 0.05$), while the level of E2 was significantly reduced ($P < 0.01$). Compared to the model group, the levels of LH and FSH in the serum of mice in the medium and high-dose MT groups were significantly reduced ($P < 0.05$), and the level of E2 was significantly increased ($P < 0.05$, $P < 0.01$). However, no statistically significant differences in hormone levels were observed between the low-dose MT group and the

model group ($P > 0.05$), suggesting that the observed effects may be related to the administered dose of MT.

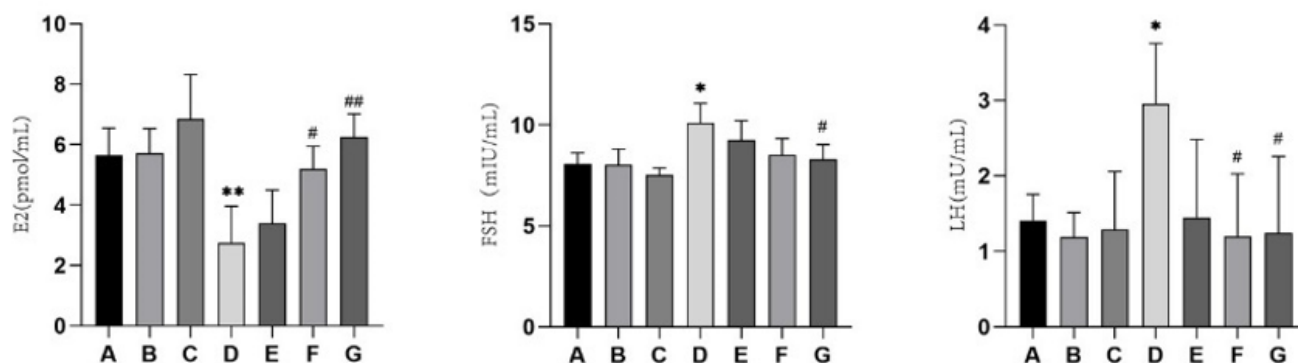


Figure 5. Comparison of serum E2, FSH, and LH in each group of mice. Note: Compared with the control group: $P < 0.05$, $*P < 0.01$; compared with the premature ovarian failure model group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Group A: Normal group, Group B: Control group, Group C: MT-only group, Group D: Premature ovarian failure model group, Group E: Low-dose MT group, Group F: Medium-dose MT group, Group G: High-dose MT group.

3.6. The effect of MT on serum MDA, SOD, and GSH-PX levels in CTX-induced POF mice

As demonstrated in **Figure 6**, there were no statistically significant differences observed between the normal group and the blank control group, compared to the control group, the serum levels of SOD and GSH-PX in the model group were significantly reduced ($P < 0.05$, $P < 0.01$), while the MDA level was significantly increased ($P < 0.05$). Compared to the model group, the serum MDA level in the high-dose MT group was significantly reduced ($P < 0.005$), and the levels of SOD and GSH-PX were significantly increased ($P < 0.005$, $P < 0.01$). However, in the low-dose MT group, only the change in SOD level was significantly different from the model group ($P < 0.005$), and in the medium-dose MT group, only the change in MDA level was statistically significant compared to the model group ($P < 0.01$).

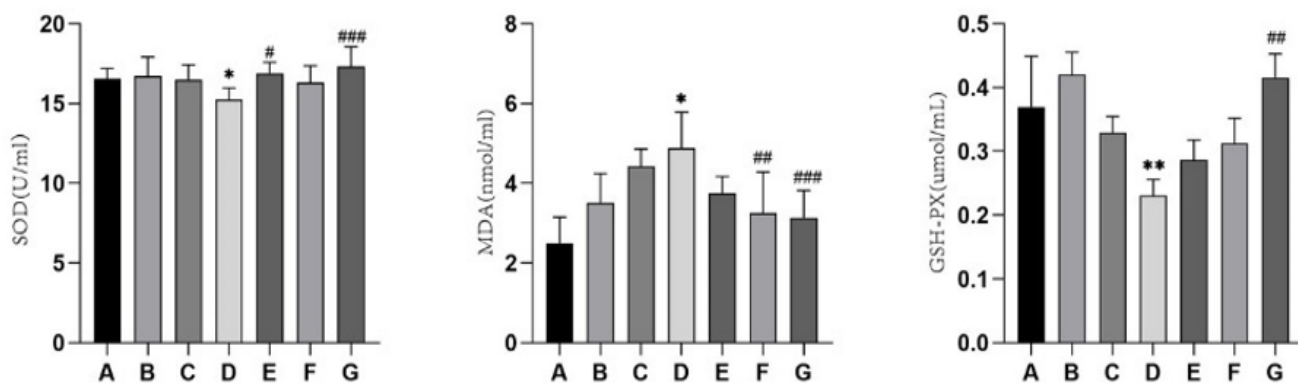


Figure 6. Comparison of MDA levels, SOD, and GSH-PX activities of mice in each group. Note: Compared with the control group: $P < 0.05$, $*P < 0.01$; compared with the premature ovarian failure model group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.005$. Group A: Normal group, Group B: Control group, Group C: MT-only group, Group D: Premature ovarian failure model group, Group E: Low-dose MT group, Group F: Medium-dose MT group, Group G: High-dose MT group.

3.7. The effect of MT on the expression of DNA damage proteins in ovarian tissue of CTX-induced POF mice

Figure 7 illustrates the results of the Western blotting assay for the expression of DNA damage proteins in the ovaries of mice in each group. The bars indicate the comparison of grey values of the four protein bands under the premise that GAPDH was used as the internal reference. In comparison with the control group, the expression of DNA damage protein γ H2AX was increased in the ovarian tissues of mice in the model group ($P < 0.05$), and the expression of p53 and p21 proteins was also significantly increased ($P < 0.01$). Conversely, the expression of γ H2AX, p53, and p21 was found to be significantly downregulated in the ovaries of mice in the low-, medium-, and high-dose MT groups ($P < 0.05$, $P < 0.01$, $P < 0.005$, respectively). Among them, the changes in the expression of various proteins were most obvious in the high-dose group, and the differences were statistically significant.

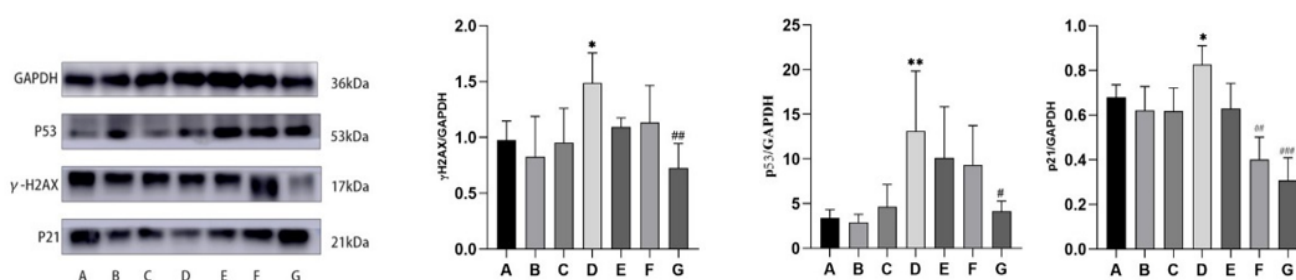


Figure 7. Comparison of the expression levels of DNA damage proteins in the ovarian tissues of mice in each group. Note: Compared with the control group: $P < 0.05$, $*P < 0.01$; compared with the premature ovarian failure model group: $\#P < 0.05$, $\##P < 0.01$, $\###P < 0.005$. Group A: Normal group, Group B: Control group, Group C: MT-only group, Group D: Premature ovarian failure model group, Group E: Low-dose MT group, Group F: Medium-dose MT group, Group G: High-dose MT group.

4. Discussion and conclusion

The global occurrence of POF is trending younger, and it poses varying degrees of harm to women's cardiovascular system, reproductive system, nervous system, and quality of life^[20]. Due to the apparent trend of younger onset of tumors, POF caused by chemotherapy drugs has gradually received widespread attention. CTX, a commonly used antineoplastic drug in clinical practice, can produce two stable toxic compounds under the action of cytochrome P450: acrolein and phosphoramidate nitrogen mustard. Acrolein has been demonstrated to induce the generation of reactive oxygen species (ROS) in a variety of cell lines within the body. An excessive accumulation of ROS within the organism has been shown to inhibit the activity of various enzymes within cells, instigate lipid peroxidation reactions, and result in DNA damage. In the female ovary, such an accumulation can precipitate accelerated follicular atresia and lead to premature depletion of the follicular pool, thus inducing ovarian failure^[21-24]. Therefore, the use of antioxidants is of great significance to the female reproductive system, as it can effectively reduce the level of free radicals in the ovaries, thereby maintaining ovarian health and delaying female ovarian aging^[25].

MT, as an antioxidant hormone that can be secreted by the human body itself, has been widely studied by scholars in recent years^[26], and the positive effects of MT on the reproductive system have been documented. For example, Jones and Pepling found that MT promotes the quality of cumulus-oocyte complexes, facilitating

the development of primordial follicles to the next morphological stage^[27]. Cruz *et al.* demonstrated that MT decreases the damage to proteins and DNA caused by oxidative stress by directly scavenging oxygen radicals and promoting glutathione synthesis^[28]. According to Matikainen *et al.*^[29], MT's ability to inhibit mitochondrial-mediated follicular apoptosis is attributed to its induction of Bcl2 in follicular granulosa cells and inhibition of caspase3 activity. Feng's^[30] research shows that melatonin can affect macrophage polarization, regulate oxidative stress and chronic inflammation, and activate ovarian germline stem cells to delay chemotherapy-induced aging in mice. Additionally, MT can indirectly activate and stimulate antioxidant enzymes and inhibit pro-oxidant enzymes. It can also reduce oxidative damage to ovarian granulosa cells by inhibiting the JNK-BCL-2-BECN1 signaling pathway to inhibit autophagy^[31].

In this study, we used CTX to construct a mouse model of POF and found that compared to the control group, the serum FSH and LH levels were elevated, while E2 levels were significantly reduced in the model group. Clinically, both LH and FSH are secreted by the pituitary gland and play a role in regulating sex hormone synthesis, promoting folliculogenesis, and corpus luteum formation^[32]. E2 is a commonly used indicator for monitoring follicular growth and development in clinical practice. It can be used to evaluate ovarian reserve function. Most patients with ovarian failure clinically have estrogen deficiency, so a decrease in E2 levels may indicate insufficient ovarian reserve function^[33]. The research conclusions of Melekoglu *et al.*^[34] and Wei *et al.*^[35] pointed out that compared with the control group, the mice in the model group had disordered estrus cycles and abnormal follicular development. The quantity of follicles at various developmental stages decreased, while the number of atretic follicles increased significantly. The disappearance of mature follicles suggests the successful establishment of a CTX-induced mouse model of POF, which is consistent with our research results^[34,35]. In this study, serum FSH and LH levels were significantly lower and E2 levels were significantly higher in all groups of mice after co-protection with MT compared to the drug control group. These findings suggest that MT improves POF in mice. Changes in the estrus cycle and ovarian morphology of mice can reflect ovarian function and ovarian reserve^[36,37]. After administering different doses of MT protection, the above conditions in mice were significantly improved, with the high-dose MT protection group showing the most significant improvement. This demonstrates that CTX is capable of inducing ovarian dysfunction and depletion of ovarian reserves in mice. Conversely, MT has been shown to restore ovarian function and preserve ovarian reserves to a certain extent, a finding that is in accordance with the research results reported by Jang *et al.*^[38].

Clinically, MDA levels serve as a means of indicating the level of lipid peroxidation in the body and, indirectly, the level of cellular damage^[39]. SOD and GSH-PX are key antioxidant molecules in the body. It has been proven that the ovaries can be shielded from oxidative stress harm by removing ROS and lipid peroxides^[40]. The experimental study revealed that, in comparison with the control group, the serum level of the oxidative index MDA was elevated, while the levels of the antioxidant indexes SOD and GSH-PX were considerably reduced in the model group mice. However, these conditions were improved in the MT-protected mice, with the high-dose group showing the most significant effect. This suggests that CTX induces oxidative stress in mice, which MT can alleviate and improve. As a structurally stable non-enzymatic antioxidant, MT has a powerful antioxidant effect that scavenges hydroxyl radicals ($\cdot\text{OH}$) and different ROS. In the body, the metabolites produced by the interaction between MT and free radicals are still powerful antioxidants. They can capture ROS through the 5-methoxy group on the indole ring, providing electrons to convert it into non-oxidizing substances, while converting itself into a low-toxicity intermediate N1-acetyl-N2-formyl-5-methoxykynurenamide. The latter has stronger antioxidant properties than MT and can scavenge a variety of ROS^[41]. 6-hydroxy MT, formed after MT

metabolism in the liver, has an antioxidant capacity 30 times that of MT. It can protect the kidneys from oxidative stress damage induced by cisplatin drugs, counteract oxidative stress caused by cyanide, and inhibit Fe^{2+} -induced lipid peroxidation reactions. Through such antioxidant cascade reactions, it becomes a highly effective antioxidant [42]. Additionally, MT also has an indirect antioxidant effect. According to research by Pandi-Perumal *et al.*, MT can enhance GSH-PX activity in the liver, lungs, and brain of rats and increase the mRNA level of SOD in tissues [43].

Furthermore, this research aimed to explore the possible mechanism of MT's antioxidant protection against premature ovarian failure caused by CTX in mice. γH2AX is a clinically commonly used marker protein for DNA damage and is one of the earliest proteins to undergo changes at DSB sites. As a signaling molecule, γH2AX recruits other DNA repair proteins to the break site to initiate the repair mechanism [44,45]. Research has demonstrated that an excessive accumulation of ROS within the body can serve as a catalyst for the onset of oxidative stress, which, in turn, can precipitate DNA damage within human tissues [46]. The results of this study showed that the expression of γH2AX protein was significantly increased in the ovarian tissues of mice in the model group compared to the control group, suggesting that CTX induced DNA damage in mouse ovaries through oxidative stress. However, the expression of γH2AX protein in the ovarian tissues of mice in the MT-protected group was significantly reduced, thereby suggesting that MT alleviated DNA damage in mouse ovaries to a certain extent. The tumor suppressor p53 is the center of the DNA damage response and a key molecule that regulates apoptosis [47]. Liu *et al.* carried out a study that demonstrated a noteworthy increase in the mRNA and protein expression levels of p53 in the ovarian tissue of rats that were suffering from POF [48]. At the same time, as a transcription factor, there is a p53-centered signal transduction network in cells. The p53 protein has been demonstrated to regulate the expression of hundreds of downstream genes, thereby triggering a variety of biological processes, including the promotion of DNA repair, the induction of cell cycle arrest, and the promotion of cell senescence and apoptosis [49]. *P21* is a transcriptional target gene of p53 [50] and an inhibitor of the cyclin E-CDK2 complex. It can arrest the cell cycle in the G1 phase during the DNA damage response, preventing it from entering the S phase and blocking the cell cycle, thus allowing more time for damaged cells to repair [51]. Zhang's research showed that MT can slow down the phosphorylation level of p53 in busulfan-induced mouse spermatogonia through the ATM-p53 signaling pathway, thus avoiding the apoptosis of spermatogonial stem cells [52]. Studies have shown that melatonin can inhibit apoptosis by down-regulating pro-apoptotic genes such as *p53*, thereby improving the quality of mouse blastocysts [53]. Zhang showed that MT can down-regulate the expression of p53 and inhibit the transcription of its downstream genes by inhibiting oxidative stress, thereby rescuing bisphenol A-induced apoptosis and autophagy of mouse Leydig cells, and ultimately alleviating bisphenol A-induced damage to the male reproductive system in mice [54]. The results of Western Blot in this study suggested that compared with the control group. The expression levels of p53 and p21 proteins were found to be significantly higher in the ovarian tissues of the model group, whereas the expression levels of these two proteins were significantly lower in the ovarian tissues of the MT-protected groups. The changes in protein expression were most significant in the high-dose group. This indicates that the protective mechanism of MT against CTX-induced DNA damage in mouse ovaries may be related to γH2AX , p53, and p21. In summary, MT can improve estrous cycle disorders, abnormal follicular development, and changes in serum hormone levels caused by CTX-induced premature ovarian failure in mice, alleviate the occurrence of oxidative stress, and protect ovarian DNA from damage. It may exert antioxidant protection by affecting the expression of proteins such as γH2AX , p53, and p21. Although this study verified this hypothesis through animal experiments, further cell experiments are needed to explore the molecular mechanism and role of MT, which will be the focus of our next research.

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Disclosure statement

The authors declare no conflict of interest.

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Analysis of the Clinical Effect of Pulmicort Respules Inhalation Combined with Cetirizine in the Treatment of Pediatric Asthma and its Influence on Inflammatory Factors in Children

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Abstract: *Objective:* To analyze the clinical value of Pulmicort Respules inhalation combined with cetirizine in the treatment of pediatric asthma. *Methods:* From December 2023 to December 2024, 82 children with asthma admitted to our hospital were randomly divided into a control group and an observation group, with 41 cases in each group. The clinical symptom relief time (shortness of breath, cough, dyspnea, lung wheezing), lung function indicators (FEV1, FVC, FEV1/FVC), inflammatory indicators (TNF- α , IL-6, IL-8), and clinical treatment effects were analyzed in the two groups. *Results:* The relief time for shortness of breath, cough, dyspnea, and lung wheezing in the observation group was shorter than that in the control group ($P < 0.05$). After treatment, compared with the control group, the levels of FEV1, FVC, FEV1/FVC, and treatment efficiency in the observation group were higher, while the levels of TNF- α , IL-6, and IL-8 were lower ($P < 0.05$). *Conclusion:* The combination of Pulmicort Respules inhalation and cetirizine oral therapy for children with asthma can shorten the improvement time of clinical symptoms, inhibit inflammation, and improve lung function.

Keywords: Pulmicort Respules; Cetirizine; Pediatric asthma; Inflammatory factors

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1. Introduction

Among pediatric diseases, asthma has become a common respiratory disorder. Recent studies have shown that with changes in people's lifestyles and the intensification of environmental pollution problems, the prevalence of childhood asthma is increasing annually^[1]. The onset of this disease not only harms the physical development of young patients but also poses challenges to their mental health. The characteristic clinical manifestations of asthma typically involve periodic wheezing, accelerated breathing, chest discomfort, or persistent coughing, and diffuse wheezing sounds can be detected in the lungs of affected children. When timely treatment is not received after

the onset of symptoms, the condition may worsen, ultimately leading to complications such as lung infections or allergic rhinitis ^[2,3]. As a new generation of antihistamines, cetirizine can inhibit specific allergens through multiple pathways, demonstrating therapeutic value in respiratory diseases ^[4]. On the other hand, Pulmicort Respules is a drug that significantly suppresses respiratory inflammation. Studies have suggested its potential efficacy in treating childhood asthma, although its precise mechanism of action remains to be further investigated ^[5]. Currently, there is no scholarly research on the combined application of these two drugs in the treatment of pediatric asthma. Based on this, the present study selected asthmatic children to receive combination therapy and analyzed its clinical value.

2. Subjects and methods

2.1. Study subjects

From December 2023 to December 2024, 82 asthmatic children were selected from our hospital and randomly divided into a control group and an observation group, with 41 cases in each group. The control group consisted of 25 males and 16 females, with an average age of 6.8 ± 1.6 years. The observation group consisted of 26 males and 15 females, with an average age of 6.5 ± 1.8 years. The baseline data of the two groups were comparable ($P < 0.05$).

Inclusion criteria: All patients met the clinical diagnosis of pediatric asthma ^[6]; their bronchodilator test was positive, and their provocation test was also positive; both the children and their families signed informed consent forms.

Exclusion criteria: Children with neurological disorders who could not cooperate well with the treatment; children with autoimmune system diseases; children with severe allergic constitutions; children who had already received other treatment regimens before enrollment.

2.2. Methods

Treatment method for the control group: The patients were treated with Pulmicort Respules. Pulmicort Respules (2 mL) were mixed with normal saline (2 mL), and the oxygen flow rate for nebulization inhalation therapy was adjusted to 6–8 L, twice a day for 14 days.

Treatment method for the observation group: The patients in the observation group were treated with cetirizine tablets plus Pulmicort Respules. Pulmicort Respules (2 mL) were mixed with normal saline (2 mL), and the oxygen flow rate for nebulization inhalation therapy was adjusted to 6–8 L, twice a day for 14 days. Additionally, cetirizine tablets (manufacturer: UCB Farchim SA; specification: 10 tablets/box; approval number: registration number H20100739) were administered. Children aged 2–5 years received 2.5 mg per oral administration, children aged 6–10 years received 5 mg per oral administration, and children over 10 years old received 10 mg per oral administration, once a day for 14 days.

2.3. Indicator analysis

2.3.1. Clinical symptom relief time analysis

The clinical symptom relief time of the two groups of children, including shortness of breath, cough, dyspnea, and lung wheezing was analyzed.

2.3.2. Lung function indicators analysis

The lung function indicators of the two groups of children before and 3 days after treatment, including FEV1, FVC, and FEV1/FVC were analyzed.

2.3.3. Inflammation indicators analysis

The inflammation indicators of the two groups of children before and 3 days after treatment were analyzed,

including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-8 (IL-8).

2.3.4. Treatment effect analysis

After treatment, if the children's clinical manifestations such as dyspnea and shortness of breath are significantly relieved, the frequency of attacks is significantly reduced, and no fine wet rales and wheezing sounds are detected in the lungs, it is considered markedly effective; if the clinical symptoms and frequency of attacks are improved after treatment, it is judged as effective; if the treatment results do not meet the above criteria, it is judged as ineffective. Except for ineffective cases, the rest are included in the effective range, and the overall effective rate is counted and compared between groups.

2.4. Statistical processing

Statistical software SPSS 26.0 was used for analysis. Measurement data is represented by mean \pm standard deviation (SD), independent sample *t*-test was used for comparison between groups, paired *t*-test was used for comparison within groups, count data was expressed as a percentage, chi-square test was used for comparison between groups, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Analysis of clinical symptom relief time in two groups of children

The clinical symptom relief time of shortness of breath, cough, dyspnea, and lung wheezing in the observation group was shorter than that in the control group, with a difference ($P < 0.05$), as shown in **Table 1**.

Table 1. Analysis of clinical symptom relief time in two groups of children (mean \pm SD)

Group	Shortness of breath	Cough	Dyspnea	Lung wheezing
Control group ($n = 41$)	4.26 \pm 0.34	5.21 \pm 1.03	3.67 \pm 0.12	4.67 \pm 0.67
Observation group ($n = 41$)	2.67 \pm 0.19	2.43 \pm 0.37	2.03 \pm 0.11	2.66 \pm 0.19
<i>t</i> -value	26.140	2.780	1.640	2.010
<i>P</i> -value	0.001	0.001	0.001	0.001

3.2. Analysis of lung function before and after treatment in two groups of children

There was no difference in lung function indicators before treatment between the two groups of children ($P > 0.05$). After treatment, the levels of FEV₁, FVC, and FEV₁/FVC were improved in both groups. Compared with the control group, the observation group had higher levels of FEV₁, FVC, and FEV₁/FVC, showing a significant difference ($P < 0.05$) as shown in **Table 2**.

Table 2. Analysis of lung function before and after treatment in two groups of children (mean \pm SD)

Group	FEV ₁ (L)		FVC (L)		FEV ₁ /FVC (%)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Control group ($n = 41$)	1.12 \pm 0.29	2.36 \pm 0.51	1.29 \pm 0.41	2.34 \pm 0.37	52.16 \pm 4.34	57.43 \pm 4.91
Observation group ($n = 41$)	1.14 \pm 0.25	3.71 \pm 0.53	1.28 \pm 0.43	3.99 \pm 0.46	52.21 \pm 4.30	62.86 \pm 5.66
<i>t</i> -value	0.335	11.750	0.107	17.900	0.052	4.640
<i>P</i> -value	0.738	0.001	0.914	0.001	0.958	0.001

3.3. Analysis of inflammation indicators before and after treatment in two groups of children

There was no difference in inflammation indicators before treatment between the two groups of children ($P > 0.05$). After treatment, the levels of TNF- α , IL-6, and IL-8 decreased in both groups. Compared with the control group, the observation group had lower levels of TNF- α , IL-6, and IL-8, showing a significant difference ($P < 0.05$) as shown in **Table 3**.

Table 3. Analysis of inflammation indicators before and after treatment in two groups of children (mean \pm SD)

Group	TNF- α (mg/ml)		IL-6 (ng/L)		IL-8 (ng/L)	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Control group ($n = 41$)	1151.34 \pm 103.29	561.29 \pm 30.37	80.67 \pm 10.12	25.64 \pm 2.19	60.37 \pm 6.94	15.16 \pm 5.34
Observation group ($n = 41$)	1150.67 \pm 102.98	500.13 \pm 19.64	80.60 \pm 11.16	15.37 \pm 1.06	60.35 \pm 6.99	9.67 \pm 1.02
t -value	0.029	10.830	0.029	27.030	0.013	6.466
P -value	0.976	0.001	0.976	0.001	0.989	0.001

3.4. Analysis of clinical treatment effects in two groups of children

Compared with the control group, the observation group had a higher effective treatment rate, showing a significant difference ($P < 0.05$) as shown in **Table 4**.

Table 4. Analysis of clinical treatment effects in two groups of children [n (%)]

Group	Effective treatment rate			
	Markedly effective	Effective	Ineffective	Total effective rate
Control group ($n = 41$)	13 (31.71)	22 (53.66)	6 (14.63)	35 (85.37)
Observation group ($n = 41$)	20 (48.78)	20 (48.78)	1 (2.44)	40 (97.56)
χ^2 -value	-	-	-	6.115
P -value	-	-	-	0.013

4. Discussion

Through systematic clinical research, it has been found that there are numerous causes of cough in children, and the pathological mechanism of cough is relatively complex. Therefore, it is crucial to accurately diagnose the etiology of the patient during the disease treatment process. Asthma, as a disease with a complex etiology, poses certain difficulties in clinical treatment^[7]. Common clinical manifestations of asthmatic children include wheezing sounds during breathing, repeated coughing, shortness of breath, and chest discomfort. Failure to provide timely and appropriate treatment not only harms the child's health status but may also negatively impact their physical development, thereby significantly reducing their quality of life. Parents need to avoid risk factors that trigger coughing in their daily lives, and once a child develops cough symptoms, they should seek prompt medical attention to prevent other complications and ensure the child's health and safety^[8].

This study found that the combination of Pulmicort Respules nebulized inhalation and cetirizine oral therapy

for asthmatic children shortened the improvement time of clinical symptoms and significantly improved lung function. This suggests that combination therapy can effectively improve lung function and clinical symptoms in these patients. The reason for this may be that cetirizine can reduce the release of inflammatory mediators such as leukotrienes and cell adhesion molecules, lower the level of vasoactive peptides, and inhibit delayed-type hypersensitivity reactions from multiple angles^[9]. Pulmicort Respules is a medication specifically designed to treat respiratory inflammation in children. Its excellent anti-inflammatory properties can rapidly alleviate symptoms in the acute phase of the disease, playing a crucial role in the treatment of pediatric asthma. Clinical studies have shown that many children have poor cooperation during treatment. To address this issue, nebulized inhalation therapy has been implemented, which not only significantly improves children's cooperation but also ensures that the medication directly targets the affected area^[10]. This therapy improves the inflammatory condition within the bronchi, enhances drug acceptability, and markedly enhances the treatment effect.

Furthermore, this study also revealed that the combination of Pulmicort Respules nebulized inhalation and cetirizine oral therapy for asthmatic children resulted in significant improvement in inflammatory markers and clinical outcomes. This may be attributed to the fact that budesonide, after nebulization, forms tiny particles ranging from 2 to 5 micrometers in diameter. These particles, propelled by oxygen, directly target the diseased sites in the capillaries, effectively blocking the accumulation of inflammatory cells and reducing the release of inflammatory mediators, thereby exerting a remarkable anti-inflammatory effect^[11]. Clinical research has found that Pulmicort Respules exhibit exceptionally strong binding affinity for glucocorticoid receptors. Coupled with its excellent solubility in water, it ensures high blood concentrations are maintained within the gel layer, thereby prolonging the duration of drug efficacy and enhancing the anti-inflammatory effect. Specifically, in the pediatric patient population, administering Pulmicort Respules via oxygen-driven nebulized inhalation allows the medication to form a "micro-reservoir" structure on the bronchial mucosa. Through this delivery method, the drug directly reaches the lungs of the child, resulting in effective deposition within the bronchi. This approach optimizes the maintenance of blood drug concentrations, greatly enhances clinical treatment efficacy, and directly inhibits the production and release of leukotrienes and arachidonic acid. This process alleviates airway resistance and facilitates improved lung function. Additionally, by optimizing the drug delivery pathway, the medication can directly target the lungs, reducing side effects during treatment and enhancing overall treatment efficacy. By adjusting the distribution of the drug within the airways, this technique further improves lung physiology, providing a more effective treatment option for pediatric patients^[12,13].

5. Conclusion

In summary, the combination of Pulmicort Respules nebulized inhalation and cetirizine oral therapy for asthmatic children can effectively improve clinical symptoms, inhibit inflammatory markers, and ultimately enhance lung function in these patients.

Disclosure statement

The authors declare no conflict of interest.

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Epidemiological Analysis of 9,064 Cases of Non-Hodgkin Lymphoma

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Abstract: *Objective:* This study aimed to investigate the epidemiological characteristics and treatment regimens of non-Hodgkin lymphoma (NHL) in China through a retrospective analysis of 9,064 NHL cases. *Methods:* Clinical data of 9,064 patients were collected from 555 hospitals in 28 provinces of China. *Results:* Among 9,064 NHL patients, there were 5,241 males (57.8%) and 3,823 females (42.2%), with a male-to-female ratio of 1.37:1. Patients aged ≥ 45 years accounted for 89.6%, with a mean age of 61.87 ± 13.30 years. The predominant NHL subtypes were diffuse large B-cell lymphoma (DLBCL, 45.2%), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL, 19.8%), marginal zone lymphoma (MZL, 13.9%), mantle cell lymphoma (MCL, 9.7%), and central nervous system lymphoma (CNSL, 4.3%). Combination therapy served as the primary treatment modality across all NHL subtypes. *Conclusions:* NHL in China demonstrates male predominance and primarily affects middle-aged and elderly populations, with combination chemotherapy remaining the mainstay therapeutic approach.

Keywords: Non-Hodgkin lymphoma; Epidemiology; Treatment regimen

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1. Background

Non-Hodgkin lymphoma (NHL) is a lymphoproliferative malignancy primarily involving lymph nodes and extranodal sites ^[1-3]. In developed countries, the most common NHL subtypes are diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL), while other subtypes exhibit incidence rates less than 10% ^[4]. In China, about 70–80% of patients are aggressive NHL subtypes. Despite the relatively high efficacy of combination chemotherapy, over 50% of patients ultimately die from NHL, underscoring its substantial societal burden ^[5]. Currently, there are few epidemiological studies on NHL in China. Therefore, this study retrospectively analyzed the data of Chinese NHL patients to investigate the epidemiological characteristics of NHL and evaluate current treatment regimens.

2. Materials and methods

Data were derived from the study named “Public Welfare Action of Health Development: Yi Xin Wei Lin.” The study included 9,064 NHL patients diagnosed in 555 hospitals of 28 provinces in China from December 2023 to July 2024. The collected data included age, sex, geographic origin, subtypes of NHL, disease stage, and treatment regimens. Descriptive analyses were performed to summarize the epidemiological features and therapeutic approaches of NHL (Table 1).

3. Results

3.1. Demographic characteristics and disease distribution

Among 9,064 non-Hodgkin lymphoma patients, 5,241 (57.8%) were male and 3,823 (42.2%) females, yielding a male-to-female ratio of 1.37:1. The age range spanned 6–95 years, with patients aged < 45 years accounting for 10.4% and those patients age ≥ 45 years comprising 89.6% of the cohort. The mean age was 61.87 ± 13.30 years. Geographically, cases were distributed across 28 Chinese provinces, with the highest frequency observed in Zhejiang Province, followed by Guangdong, Jiangsu, Sichuan, and Shandong, while fewer cases came from northeastern China (Figure 1).

The NHL subtypes identified included diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), mantle cell lymphoma (MCL), central nervous system lymphoma (CNSL), marginal zone lymphoma (MZL), Waldenström macroglobulinemia (WM), and other rare subtypes. DLBCL accounts for the majority of the patients (45.2%), followed by CLL/SLL (19.8%), MZL (13.9%), MCL (9.7%), CNSL (4.3%), follicular lymphoma (FL, 3.7%), WM (0.5%), and other subtypes (2.8%).

3.2. Disease status and treatment patterns

Disease progression status was categorized as treatment-naïve (48.6%), relapsed (45.0%), or refractory (6.4%). Regarding therapeutic approaches, combination therapy constituted the primary regimen (6,080 cases, 67.9%), while monotherapy was administered in 2,868 cases (32.1%).

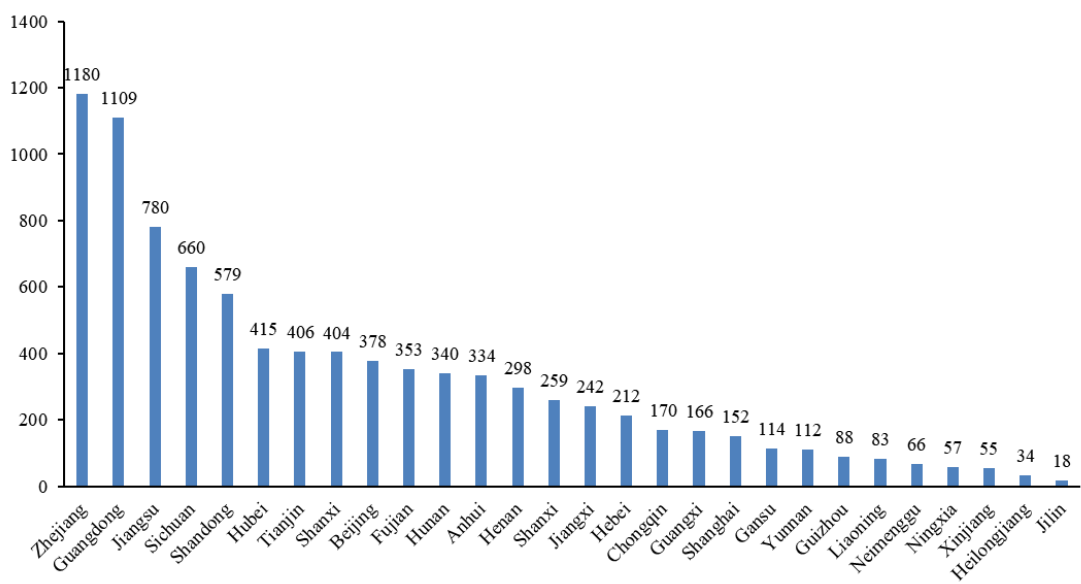


Figure 1. Geographic distribution of 9,064 non-Hodgkin lymphoma cases in China

Table 1. Descriptive analysis of baseline characteristics in patients with non-Hodgkin lymphoma

Parameters	All (<i>n</i> = 9,064)
Age	
Mean ± SD	61.87 ± 13.30
Median (Q1, Q3)	63.00 (54.00, 71.00)
Min, max	6.00, 95.00
Age group	
Age < 30	200 (2.2%)
30 ≤ age < 45	747 (8.2%)
45 ≤ age < 60	2573 (28.4%)
60 ≤ age < 75	4109 (45.3%)
Age ≥ 75	1435 (15.8%)
Gender	
Male	5241 (57.8%)
Female	3823 (42.2%)
Types of lymphoma	
DLBCL	4099 (45.2%)
CLLSLL	1795 (19.8%)
MZL	1261 (13.9%)
MCL	882 (9.7%)
CNSL	392 (4.3%)
FL	336 (3.7%)
WM	46 (0.5%)
Others	253 (2.8%)
Disease stage	
Initial treatment	4409 (48.6%)
Recurrence	4076 (45.0%)
Intractable	579 (6.4%)
Treatment plan	
Monotherapy	2868 (32.1%)
Combination therapy	6080 (67.9%)
Missing	116 (1.3%)

DLBCL: Diffuse large B-cell lymphoma; CLL/SLL: Chronic lymphocytic leukemia/small lymphocytic lymphoma; MCL: Mantle cell lymphoma; CNSL: Central nervous system lymphoma; MZL: Marginal zone lymphoma; WM: Waldenström macroglobulinemia; FL: Follicular lymphoma;

4. Discussion

This real-world study data came from 9,064 NHL patients across 28 Chinese provinces. Results of the study revealed a slight male predominance (male-to-female ratio: 1.37:1), with middle-aged and elderly individuals constituting the majority (89.6% aged ≥ 45 years). Demographic characteristics including age and gender distributions across NHL subtypes aligned with prior reports ^[6].

DLBCL was the most prevalent subtype (45.2%), consistent with domestic studies but slightly higher than rates reported in Western countries ^[6-9]. Two-thirds of DLBCL patients achieved favorable outcomes with the R-CHOP regimen (cyclophosphamide + doxorubicin + vincristine + prednisone). Preliminary trials of Bruton's tyrosine kinase (BTK) inhibitor-based combination therapies demonstrated promising efficacy and safety in DLBCL treatment ^[10].

CLL/SLL accounted for 19.8% of the total patients, ranking second in prevalence, significantly higher than rates of 6.39% reported by Li *et al.* ^[9] and the studies of Western countries (7–10%) ^[11]. Head-to-head studies showed superior progression-free survival and overall response rates with zanubrutinib versus ibrutinib ^[12]. A U.S. real-world evidence further highlighted zanubrutinib's enhanced safety profile, particularly lower cardiovascular adverse events than ibrutinib and acalabrutinib ^[13].

MZL comprised 13.9% of all the cases. This heterogeneous subtype encompassing mucosa-associated lymphoid tissue (MALT), splenic, and nodal MZL, requires tailored treatment strategies based on subtypes and stages. As an indolent lymphoma, MZL generally exhibits favorable responses to conventional therapies and prolonged survival.

MCL represented 9.7% of all the cases. Despite classification as a chronic B-cell lymphoproliferative disorder, MCL often demonstrates aggressive progression. Median survival with chemotherapy alone remains limited to 3–4 years ^[14]. Standardized therapies are lacking, and early identification of high-risk patients is critical to optimize outcomes ^[15,16].

CNSL accounted for 4.3% of all the cases, predominantly DLBCL histology. Multidisciplinary collaboration is essential for optimal management. While whole-brain radiotherapy historically achieved $> 80\%$ response rates, high-dose methotrexate (HD-MTX) based combination were the first-line treatment regimen for primary CNS lymphoma (PCNSL) due to rapid relapse risks ^[17].

FL, the most common indolent B-cell lymphoma, constituted 3.7% of all the cases. Patients with relapsed/refractory FL after multiple lines of therapy face poor prognoses. Emerging strategies, including CAR-T cell therapy and bispecific antibody-based regimens, show efficacy in refractory cases, though optimal sequencing and combination approaches require further investigation ^[18].

WM, a rare indolent mature B-cell lymphoma, represented 0.5% of all the cases. The first-line treatment for symptomatic patients depends on factors such as age, clinical manifestations (e.g., cytopenias, organomegaly), and eligibility for autologous stem cell transplantation (ASCT). Rituximab-based chemotherapy remains the primary treatment ^[19].

5. Conclusion

This nationwide, large-scale retrospective analysis delineates the epidemiological distribution and therapeutic landscape of major NHL subtypes in China, providing critical insights for future research and clinical strategies.

Disclosure statement

The authors declare no conflict of interest.

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The Spatial Location of Chromosomes in Dividing Cells and the Relative Stability of Chromosome Spatial Structure

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Abstract: The stability and evolution of human genetics depend on chromosomes and chromosome-chromosome interactions. We wish to understand the spatial location of chromosomes in dividing cells in order to understand the relationship between chromosome-chromosome interactions and to further investigate the role of chromosomes and their impact on cell biological behavior. In this study, we explored the relative spatial positional relationships of chromosomes [t(9;22) and t(15;17)] in B-ALL cells by using the three-dimensional DNA fluorescent *in situ* hybridization (3D-FISH) method. The results showed that chromosomes [t(9;22) and t(15;17)] showed relatively stable spatial relationships. The relative stability of the spatial location of chromosomes in dividing cells may be relevant to disease.

Keywords: Chromosome; Human genetics; Chromosome territory; 3D-FISH

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1. Introduction

During interphase, each chromosome occupies a separate nuclear space to form a chromosome territory (CT) ^[1-3], which has been shown to have non-random radial nuclear distributions ^[4-6]. The spatial conformation of CTs in the nucleus is non-random ^[7]. Several studies in recent years have demonstrated a link between the spatial conformation of the genome and some basic biological processes (gene recombination, gene expression, differentiation, etc.) ^[8,9]. Chromosomes are considered natural units of subdivision of the complete genome. Karyotype abnormalities are often present in several clinical conditions such as hematologic tumors ^[10,11], malformations ^[12], infertility ^[13], etc. An interesting phenomenon is that most of the karyotype abnormalities are single points, which seems to suggest the timing of the karyotype abnormalities. Chromosomes adjacent to each other in the nucleus may be more likely to undergo translocation than those relatively distant from each other, suggesting the relevance of spatial location for the occurrence of karyotypic abnormalities. During mitosis, chromosomes can easily be seen as highly condensed structures in cells ^[14], but their intracellular location and the spatial relationships between chromosomes are poorly understood. Chromosome structure and arrangement in cells are only well observed in the interphase of cells ^[15].

Previously, we chose to analyze the data of 66,919 valid karyotype abnormalities in the National Cancer Institute database (April 26, 2017) ^[15]. We analyzed the karyotype abnormalities occurring between chromosomes in the karyotype abnormality data and found a high frequency of karyotype abnormalities occurring. Plus, many karyotypic abnormalities were isolated or rare. The data collected on karyotypic abnormalities (karyotypic abnormalities between chromosomes) were analyzed. We followed two models for this, a planar model of chromosomes on the equatorial plate of the medium-term cell and a circular or a foveal arrangement.

Fluorescent *in situ* hybridization (FISH) is an analytical detection technique. It obtains information on the status of multiple chromosomes or multiple genes by hybridizing fluorescently labeled nucleic acid probes with nucleic acid sequences and further analyzing the probe signals with the aid of fluorescence microscopy ^[16]. FISH has the advantages of a short detection cycle, high stability, high sensitivity, and stable probes that can be stored for a long time, etc. It is mainly used clinically for tumor diagnosis, prognosis assessment, and guidance of targeted drug therapy ^[17]. Three-dimensional (3D)-FISH is a genetic sequence-specific biomarker technique based on the traditional *in situ* hybridization technique ^[18,19]. It can specifically label specific sequences of genetic material in the nucleus without destroying the cell structure. It is because of this 3D-specific labeling feature that 3D-FISH has been widely used in the study of the 3D spatial conformation of genetic material.

In this study, we stained, scanned, and 3D reconstructed the chromosomes of mid-stage cells using the 3D-FISH technique to observe the spatial state of chromosomes in mid-stage cells and to explore the spatial location of chromosomes within the cells and the spatial relationship between chromosomes. We aimed to further understand the chromosomes and the biological behavior between them.

2. Materials and methods

2.1. Samples

BALL-1 and HL-60 cells were purchased and cultured as recommended by ATCC and DSMZ, respectively. Then, they were selected: karyotype t(9;22) and t(15;17), respectively. Another human BALL-1 cell line (male) was used as a control. The cell lines were all treated as interphase cell lines. Each type of cell line was divided into male and female and was predominantly male. Ethical approval was given by the medical ethics committee of Soochow

2.2. Cell culture and preparation

All cells were terminated at interphase and were hypotonicized (colchicine was added at 0.05 g/mL for one hour). Subsequently, cells were fixed with 3:1 methanol:acetic acid three times. Conventional filming is to observe the karyotype of chromosomes, which requires cell fragmentation and natural dispersion of chromosomes; while this experiment requires observation of the structure and arrangement of chromosomes in the cells, the structural integrity of the cells and effective dispersion of the cells are required. Therefore, we adjusted the height of the drop film and lowered it by about one-third.

2.3. Probe preparation and selection

The type of probe was a fully stained probe purchased commercially (Creative Bioarray, Shirley, NY, USA). Color crosstalk is an important factor affecting the experimental results (especially when crosstalk is between adjacent chromosomes), four fluorochromes per group were chosen to label the chromosomes. For this reason, we used different schemes to mark different chromosomes with different possible distances as fluorochromes and chromosome corresponding marks based on the results of the preliminary data analysis, reducing the chance of crosstalk between possible adjacent chromosomes. In this study, the corresponding cell lines (target cell lines) were hybridized with the probes separately, while cell lines with normal karyotypes were hybridized separately as controls using two sets of probes. Two sets of chromosomes were selected: Group 1 [t(9; 22), sex chromosomes] and Group 2 [t(15;17), sex chromosomes]. Each chromosome was color-coded for single-color fluorescence separately (Table 1).

Table 1. Probe fluorescence color and group

Lable	Abs. (nm)	Em. (nm)	Group 1	Group 2
DAPI/Aqu	405	~470	——	——
Green	488	~510	15	22
Red	543	~570	X	X
DIG	594	~615	17	9
BIOTIN	639	~660	Y	Y

2.4. 3D-FISH

2.4.1. Pretreatment

Frozen sections were removed from the slides stored at -80°C, and the tissues were fixed by immersing the slides in ice-cold 4% paraformaldehyde (PFA) for 15–30 minutes at 4°C. The slides were then rehydrated for 5 minutes each in 100%, 90%, and 70% ethanol, followed by washing in distilled water for 1 minute and in PBS for 5 minutes. Subsequently, the slides were heated in distilled water at 100°C for 15 minutes, treated with pepsin solution for 3–15 minutes at 37°C, and washed in PBS. Finally, the slides were dehydrated by incubating them in pre-cooled 75%, 85%, and 100% ethanol for 1 minute at each concentration, and then air-dried.

2.4.2. Co-denaturing and hybridization

The denatured probe was applied to the slide (10 μ L of probe for each slide). A coverslip was immediately applied and sealed with rubber cement. The slides were denatured at 80°C for 5 minutes and hybridized at 37–42°C overnight (~20 hours).

2.4.3. Washing

Rubber cement and coverslips were removed, and to prevent drying, the slides were temporarily kept in washing solution at ambient temperature to facilitate the removal of coverslips. The slides were then immersed in SSC/0.3% NP-40 at $74 \pm 1^\circ\text{C}$ for 2–5 minutes, followed by immersion in 2X SSC/0.3% NP-40 at room temperature for 5 minutes. Subsequently, the slides were dehydrated by incubation in 75%, 85%, and 100% ethanol for 1 minute at each concentration. Finally, the slides were air-dried in darkness.

2.4.4. Counterstain

A volume of 20 μ L of antifade solution with DAPI was placed on the surface, and a cover glass was positioned over it. Any air bubbles that may have formed were carefully removed.

2.5. Confocal microscopy

2.5.1. Microscopy

Confocal imaging was performed on a Zeiss LSM 980 laser scanning confocal system with a Plan-Apochromat $\times 63/1.40$ -NA DIC M27 oil-immersion objective.

2.5.2. Confocal scanning method

The pinhole during all the image acquisition was opened at 1 Airy unit. Laser powers and detector gain were optimized for each sample. An Airyscan II detector was also used for image acquisition. The system was controlled by the ZEN software (Zeiss, blue edition). A 405 nm diode laser was used for DAPI excitation; a 488 nm diode laser was used for green probe excitation; an A 543 nm diode laser was used for red probe excitation; a 594 nm diode laser was used for DIG-labeled probe excitation; a 639 nm diode laser was used for BIOTIN-labeled probe excitation. Between the Red probe and the DIG-labeled probe, Zeiss' unique spectral resolution method was used to eliminate the risk of color crosstalk. Image processing was performed by ZEN Airy scan processing using automatic deconvolution parameters; the 3D visualization of images was processed by the ZEN 3D module, and the most appropriate surface parameters were processed for each sample.

2.5.3. Observation of images

The XY chromosome was used as an anchor point to see the other chromosomes themselves and their spatial location in relation to the XY chromosome.

3. Results

From **Figures 1 to 3**, we can see the relatively stable state of the chromosomes. The chromosome arrangement of different cells of the same cell line of the same set of probe hybridization is relatively stable: the spatial position of the three sets of chromosomes can be seen to be relatively stable. The spatial positions of chromosomes in different

cell lines of the same cell type also have similar spatial position relationships. The spatial position relationships of the chromosomes within the cells observed for each set of probe hybridization for the target cell line and the cell line with normal karyotype are also relatively stable.

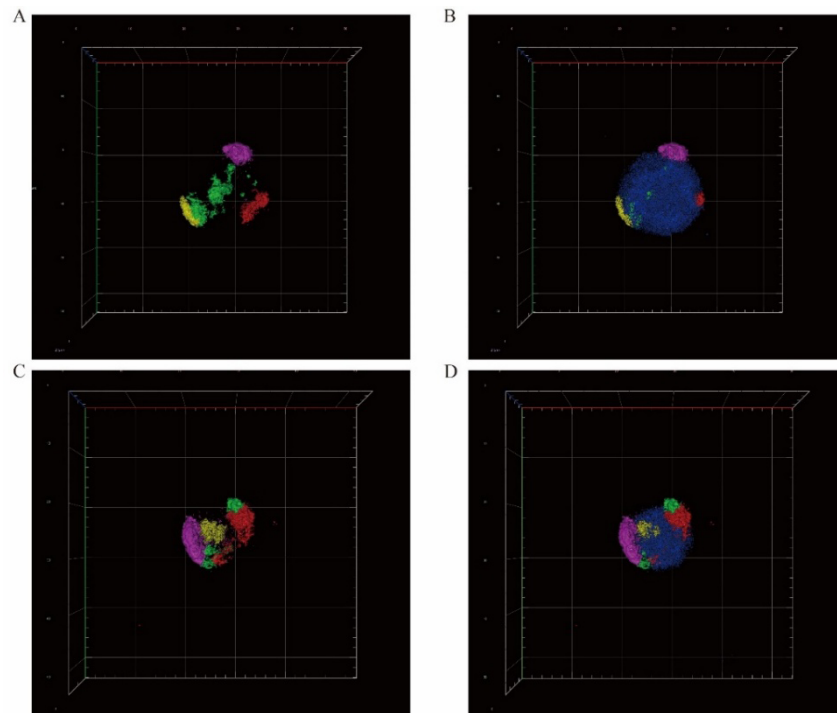


Figure 1. 3D-FISH visualization of chromosomes in normal human (male) nuclei of BALL-1 cell. A, C: chromosome 9 (DIG); 22 (Green); X (Red); Y (BIOTIN); B, D: chromosome 9, 22 with DAPI (Aqua)

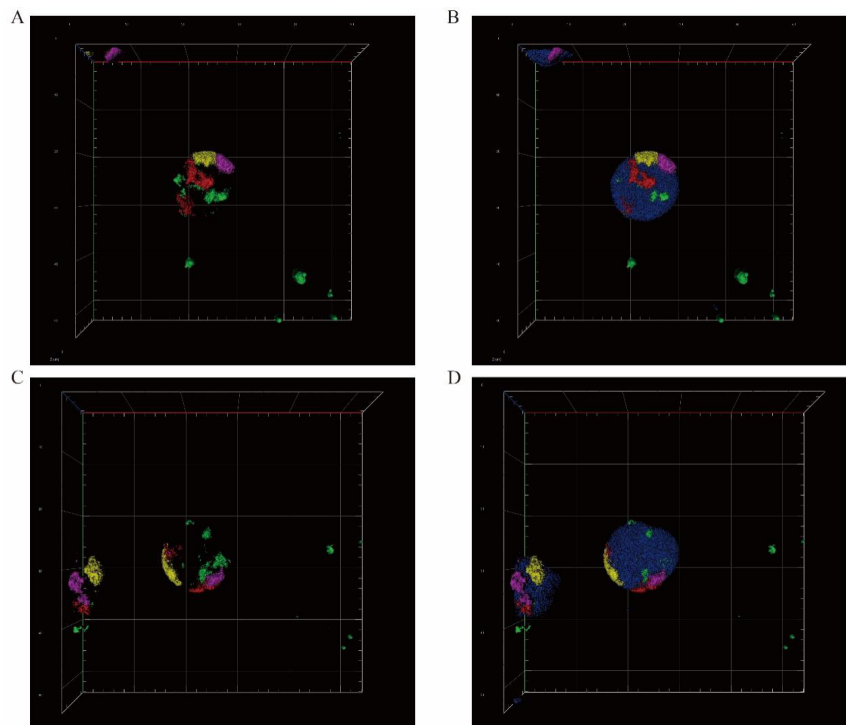


Figure 2. 3D-FISH visualization of chromosomes in case 1's (male) cell nuclei. A, C: chromosome 9 (DIG); 22 (Green); X (Red); Y (BIOTIN); B, D: chromosome 9, 22 with DAPI (Aqua)

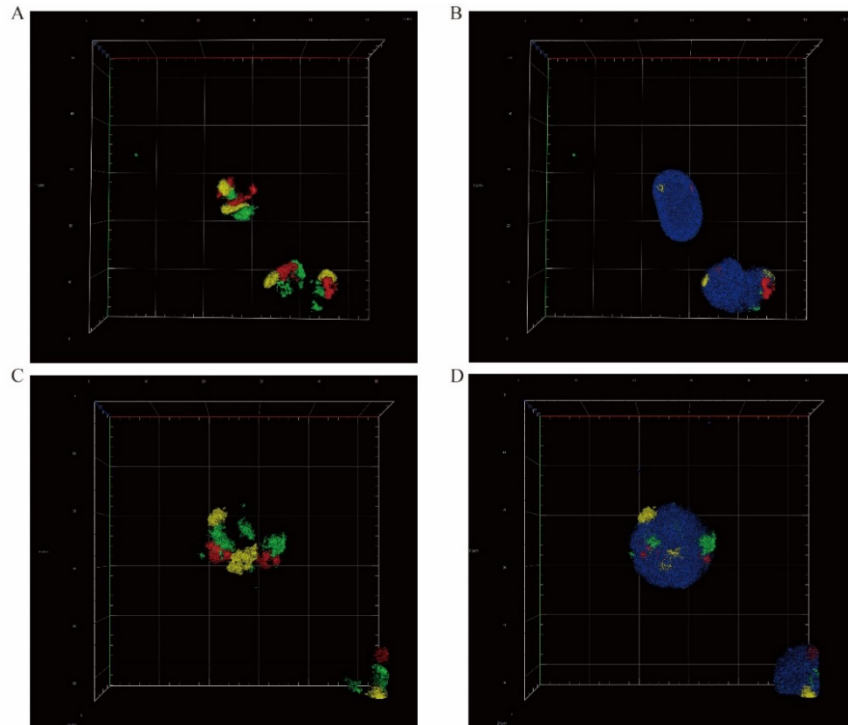


Figure 3. 3D-FISH visualization of chromosomes in case 2's (female) cell nuclei. A, C: chromosome 9 (DIG); 22 (Green); X (Red); Y (BIOTIN); B, D: chromosome 9, 22 with DAPI (Aqu)

4. Discussion

The orientation of the cells, as they drip onto the slide, is random due to the cells being round or oval. The equatorial plates within the cells, and the chromosomes in them, do not maintain a neat and uniform orientation, which makes it somewhat challenging to compare the spatial position of the chromosomes with each other. At the same time, because the cells are affected by the coverslip during the filming process, the structure of the cells is intact but flat, so the chromosomes inside the cells are somewhat squeezed and there is a certain displacement of the spatial position relationship of the chromosomes, which is an overall displacement that does not affect the position relationship. It also does not affect our results and the judgment of the results. Since the cells are approximately round or oval, the cells are filmed by drip film, and the spatial position of the cells currently is random. This has an impact on the processing of information after scanning, especially the confirmation of the spatial position of chromosomes after 3D reconstruction. By adjusting the three-dimensional information of the cells, we can understand their positional relationships more intuitively.

The two sets of probes hybridized different cell lines separately, and both observed relative stability in the spatial positional relationships between chromosomes within the cells. Each set of probes hybridized the target cell line and the cell line with normal karyotype, and their observed spatial position relationship of chromosomes within the cell was also relatively stable. This is a remarkable and meaningful phenomenon. It strongly suggests the stability of the spatial position of chromosomes within the medium-term cell cells. However, we need the support of more data to further refine and enrich our observations.

There are two sets of chromosomes in the cell, X and Y chromosomes as sex chromosomes, and it is very interesting how they are assigned to one of them. More observations are needed to further understand the

implications.

Chromosomes 9 and 22 and chromosomes 15 and 17 are stably present and arranged near each other. This is also true in the same cell line and different cell lines. In the chronic granulocytic leukemia cell line or the normal karyotype cell line, their pattern is consistent. Based on the present phenomena, it appears that the chromosomes may be regularly arranged on the equatorial plate in intermediate cells, as is the case for chromosomes 9 and 22 and chromosomes 15 and 17. However, this requires more data and observations of different probes for further verification.

The relative spatial positions of chromosomes 9, 22, and X and Y, as well as 15, 17, and X and Y, correspond to each other, as the long and short arms of the chromosomes may be in free suspension within the cell and positioned by the chromosome's mitoses. This causes the non-uniform overall position of chromosomes, with small drifts of chromosomes in a relatively stable position. There may be other chromosomes caught in between the individual chromosomes. It is just that they are not marked and displayed for us to observe, thus more experiments are needed to observe and understand this.

The arrangement of chromosomes on the equatorial plate in human intermediate somatic cells is stable and regularly arranged; X and Y chromosomes are arranged correspondingly on the equatorial plate in cells in females, and X chromosomes appear in pairs as sex chromosomes. In male cells, the sex chromosomes are X and Y chromosomes, which are currently seen to appear in proximity. The arrangement of the segregation of X and Y chromosomes needs to be further investigated.

The XX chromosomes are the two pairs of sex chromosomes in females, which naturally belong to two different sides of the equatorial plate of the cell, in a corresponding arrangement. In males, the sex chromosomes are the X and Y chromosomes, and their arrangement is a very interesting expectation. The current observation shows that the position of the X chromosome about the Y chromosome and other groups of chromosomes (15, 17; 9, 22) is also relatively fixed. The prediction is that the two sex chromosomes X and Y may also be arranged in correspondence.

In several cell lines of chronic granulocytic leukemia performed so far, we found that chromosomes 9 and 22, which constitute Ph1, can originate from the same ring or different rings. This is a significant phenomenon, but its clinical significance is not yet clarified, thus we need to do more data accumulation and clinical observation. The translocation of chromosomes 15 and 17 also requires further research.

5. Conclusion

The relative stability of the spatial location of chromosomes in interphase cells may be associated with disease.

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Disclosure statement

The authors declare no conflict of interest.

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