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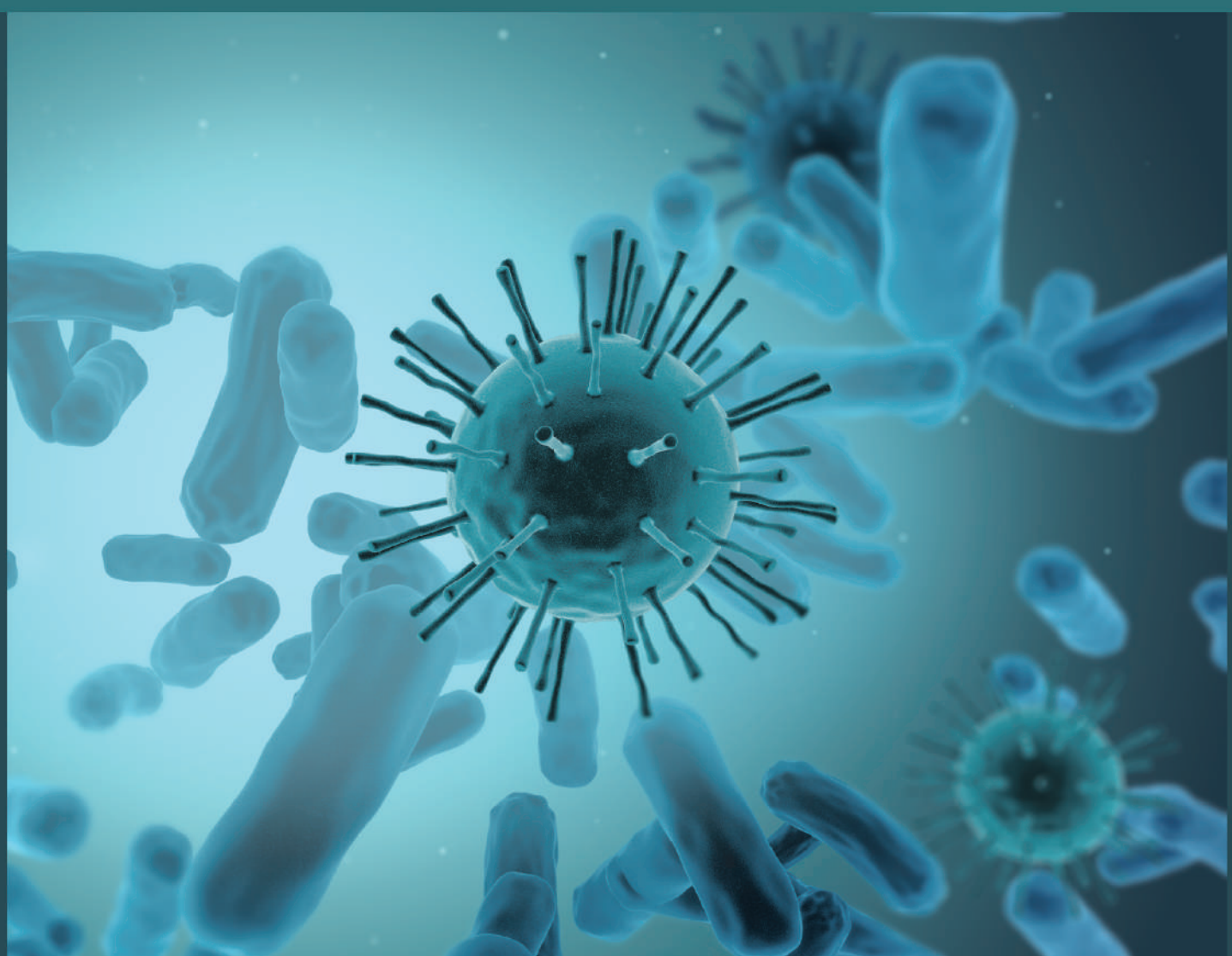

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ARTICLE

Signaling Pathways Associated with Cancer Stem Cells Play a Significant Role in Immunotherapy Resistance

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ABSTRACT

Cancer stem cells (CSCs) are a subpopulation of tumor cells with properties of self-renewal, pluripotency, plasticity, and differentiation, and are associated with various aberrantly stimulated signaling pathways. They are responsible for tumor recurrence, distant metastasis, and drug resistance, thus inducing poor prognosis. Immunotherapy has achieved encouraging results. However, the resistance associated with its clinical application is a persistent problem in clinical and scientific researches. Increasing evidence shows that signaling pathways associated with CSCs mediate immunotherapy resistance. This review highlights the link between them, and focuses on the underlying mechanism so as to provide potential strategies and approaches for the development of new targets against the immune resistance challenge.

1. Introduction

Cancer is considered a heterogeneous disease due to the subsets of cells with distinct phenotypes and functions [1-3]. A small group of cancer cells with stem-like abilities are found in almost all untreated human malignancies. These cells are termed "cancer stem cells" (CSCs) based on their biological similarities with normal stem cells found in the same tissue [1,4]. CSCs were first identified in acute myeloid leukemia (AML), and later were also found in numerous solid tumors, such as breast, thyroid, prostate, brain, lung, colon, melanoma, liver, and stomach cancers [5-15]. CSCs have characteristics of self‐renewal, differentiation, quiescence, and potential function to build their heterogeneity and induce cancer growth [16,17].

With the improved detection and treatment of cancer, some primary tumors can be completely cured after surgery. However, patients with advanced, metastatic, and/or recurrent tumors are in need of standard therapies, such as chemotherapy, radiotherapy, and molecular targeted therapy. Mounting studies indicate that these ther-

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apies target the relatively differentiated and proliferating cancer cells. While these CSCs are mostly dormant and have been demonstrated to contribute to many clinical therapies, subsequently leading to tumor relapse, metastasis recurrence, and poor prognosis [18,19]. The underlying mechanisms of resistance to therapies by CSCs are explained by the overexpression of anti-apoptotic proteins, augmented DNA-repair capacity, aberrantly stimulated signaling pathways, elevated anti-oxidant proteins, activated epithelial to mesenchymal transition (EMT) program, and adapted metabolism under hypoxia conditions. In addition, the capability of CSCs to evade the immune system make it more difficult to overcome the therapy resistance [4, 20-24].

Recently, immunotherapy has emerged as a promising treatment for cancer patients and regained global attention [25]. Immune checkpoint inhibitors (ICIs) have been approved for the treatment of various aggressive cancers [26-30]. Despite the unprecedented favorable outcome observed with immunotherapies, the response rates remain low, ranging from 15-40% varying from cancer types [31-33]. A majority of the patients do not benefit from the ICIs, mainly because tumors can escape immunosurveillance and elimination by avoiding the detection of the immune system or suppressing immune responses. Like tumor cells, CSCs also have developed diverse strategies to escape the immune protection , including loss of tumor antigen expression, reduce of immune recognition via genetic or nongenetic alterations , enhancement of tolerance to immune cytotoxicity, and promotion of a immunosuppressive microenvironment [34]. Furthermore, previous studies have demonstrated that CSCs are associated with immunotherapy resistance in various cancer types [35,36]. However, the related signaling pathways remain poorly understood. Herein, we summarized the signaling pathways of associated with CSCs with regard to their mechanistic regulation networks and their roles in immunotherapy resistance.

2. The Related Signaling Pathways of CSCs Implicated in Immunotherapy Resistance

Several cellular signaling pathways, such as Notch, Hedgehog (Hh), Transforming growth factor-beta (TGF-β), WNT/β-catenin, EGFR, NF-κB, HIF-1α, MAPK, PTEN/PI3K, and JAK/STAT [37-39], have been described to play a vital role in the induction and maintenance of stemness in CSCs. Among these, TGF-β, WNT/β-catenin, Hippo, HIF-1α, and Hh pathways are associated with immunotherapy resistance (Figure 1).

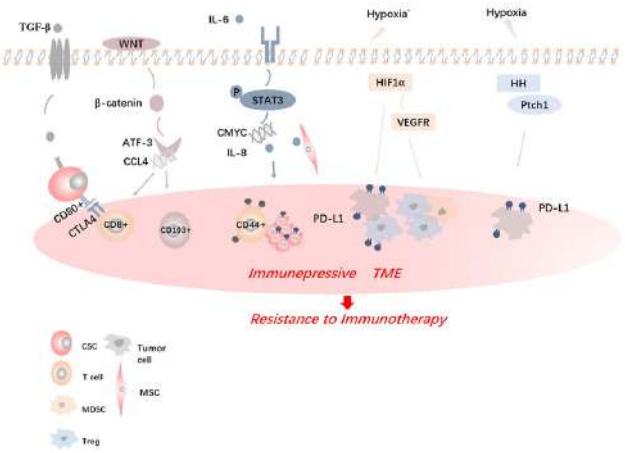

Figure 1. Signaling Pathways of Cancer Stem Cells in Resistance to Immunotherapy

Note: Collectively, TGF-β, WNT/β-catenin, Hippo, HIF-1α, and Hh pathways are associated with immunotherapy resistance.

2.1 TGF-β-responding CSCs Via CD80 Activation are Responsible for Immunotherapy Resistance

TGF-β signaling plays a dominant role in mediating EMT in CSCs [40-43]. It becomes phosphorylated upon binding to the TGF-β receptor. Subsequently, SMAD2/SMAD3 is activated and composes into a complex with SMAD4. This complex translocates to the nucleus as a transcription factor, leading to the expression of target genes implicated in stemness and invasion property of cancer cells [44]. The TGF-β signal can also remodel the tumor microenvironment (TME) by inhibiting T cell differentiation and activity, thus resulting in poor prognosis [45,46].

Two studies have identified the TGF-β signaling is a determining factor of T cell rejection and poor response to ICIs [45,47]. Furthermore, in mouse models, promising preclinical evidence showed that the combination of TGF-β inhibitors and ICIs can facilitate T cell infiltration into the tumor center, extensively promoting anti-tumor immunity [48]. A similar model was designed for squamous cell carcinoma. It revealed that the CSCs equipped with the surface CD80 not only have the power to resist immunotherapy by stimulating direct dampening of cytotoxic T lymphocyte (CTL) activity but also accelerate tumor growth. In contrast, the loss of CD80 can restore CTL proliferation to a greater extent than ICIs, making CSCs vulnerable and diminishing the immune-related tumor relapse. This is because CD80 is only activated in TGF-β-responding CSCs, and its expression could be influenced by TGF-β signaling. The single-cell RNA sequencing (RNA-seq) of TGF-β-responding CSCs shows that they are superior at resisting CTL responses and constitute the root of tumor recurrence [49]. The role of TGF-β responding CSCs in assisting cancer

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immune escapes has also been demonstrated in bladder and colon cancer after conventional PD-L1 immunotherapy [47,48,50]. These results indicate that the combination of TGF-β inhibitors and ICIs might be effective in targeting the CSCs to overcome immunotherapy resistance.

2.2 Tumor-intrinsic Active WNT/β-catenin Signaling Results in T-cell Exclusion

WNT signaling plays a substantial role in keeping CSCs in a undifferentiated and self‐renewal state; therefore, the activated WNT signaling is associated with cancer occurrence [16]. In colon cancer, WNT/β‐catenin can be activated by protein‐4 (AP4), thereby increasing the number of CSCs and modulating their homeostasis [51]. In lung cancer, β‐catenin signaling contributes to the maintenance of CSC phenotype, and stemness [52,53]. The activation of WNT signaling via the hepatocyte growth factor (HGF) promotes the transition of cancer cells into CSCs [54, 55].

The role of WNT signaling in immune escape has recently been discovered. The molecular analysis of human metastatic melanoma samples shows that the activated WNT signaling is correlated with T-cell exclusion [56]. Similarly, β-catenin appears to inhibit CTL activation [57]. Mechanistically, previous reports have indicated that CCL4 can induce T-cell infiltration [58,59]. Meanwhile, the WNT/β-catenin signaling suppressed the CCL4 gene expression via ATF3-dependent transcriptional expression, resulting in immune evasion [60]. In a melanoma mouse model with constitutively high β-catenin activity, the failure of T-cell initiation against tumor antigens is mainly attributed to the decreased infiltration of $CD103^+$ dendritic cells [61]. The restoration of dendritic cell recruitment into the tumor via injection can enhance anti-PD-L1/CTLA4 therapy. Moreover, the upregulation of IL-12 by β-catenin signaling can also modulate and impair the dendritic cell function [60]. Similarly, in colon cancer, the inhibition of β-catenin activity of increases CD8+ T cells and $CD103^+$ levels in tumor area. β-catenin signal may mediate immunotherapy resistance of colon cancer [62]. Collectively, the manipulation of Wnt/β-catenin signaling pathway combined with ICIs might represent a novel therapy for cancer, further studies investigating the interaction between tumor intrinsic WNT/β-catenin signaling and immunotherapy are expected.

2.3 STAT3 Signaling-mediated IL-8 Derived from Gastric Cancer Mesenchymal Stem Cells (GCMSCs) Increases PD-L1 Expression to Resist CD8+T Cell Cytotoxicity

Signal transducers and activators of transcription (STAT) factors and the receptor-associated JAK kinases, are the downstream effectors of both extrinsic and intrinsic signals [63,64]. Tyrosine-phosphorylated (YP)-STATs compose into an active dimer and control target genes expression in the nucleus [65]. Excessive activation of STAT3 was reported to play many roles in cancer cells, including the promotion of cancer cell survival, proliferation and tumor angiogenesis, down-modulation of anti-tumor immune responses, enhancement of tumor recurrence and metastasis by inducing EMT, and increasing the number of CSCs. Finally, STAT3 activity can induce CSC features in solid tumors [66-68]. Therefore, STAT3 is regarded as an oncogene and a target for anti-cancer treatments

The activation of STAT3 signal is involved in the modulation of PD-L1 expression [69,70]. IL-8 derived from the GCMSCs induces PD-L1 expression in gastric cancer (GC) cells [71]. In contrast, IL-8 inhibition weakened the protective effects of GCMSCs on GC cells against CD8+ T cell cytotoxicity. The inhibition of IL-8 derived from GCMSCs may suggest a potential strategy to sensitize PD-L1 antibody therapy in GC. In addition, the combinative blockade of multiple cytokines with ICIs in the future may have the potential to overcome the immunotherapy resistance induced by the high expression of PD-L1. Furthermore, CD44+ cells are also found to have an EMT property and are less immunogenic. CD44+ cells were observed to have a high inducible expression of PD-L1 and associated with the phosphorylation of STAT3. Therefore, CD44+ cells are characterized with drug immunotherapy resistance. Inhibition of STAT3 could decrease the expression of PD-L1 on CD44+ cells and selectively enhance the immune responses [72]. Interestingly, subsets of CSCs with an EMT phenotype are low immunogenicity due to elevated PD-L1 expression, driven by the constitutive phosphorylation of STAT3 [72,73]. Considering these evidences, STAT3 expression may decrease the therapeutic efficacy of ICIs, and the combination of immunotherapy with STAT3 inhibitors may be a promising strategy to effectively suppress malignant tumors. Further investigation of the specific function of STAT3-regulated PD-L1 expression on the surface of cancer cell and CD44+ cells will be required to fully understand the intriguing link between immune escape and signaling pathways associated with CSCs.

2.4 HIF Signaling Drives the Expression of PD-L1 and Induces the Immunosuppressive Tumor Microenvironment

Hypoxia is one of the most common features of the TME driving the aggressiveness of tumors [74]. Hypoxic remodeling is mostly regulated by hypoxia-inducible

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factors (HIFs) [75]. Three HIF-α family proteins are described in humans: HIF-1α, -2α, and -3α. Among these, HIF-1α expression up-regulation is well understood and found in many tumors, such as prostate cancer, breast cancer, colon cancer, and hemangioblastoma [76]. Activated HIF pathway can initiate genes associated with vasculogenesis, drug resistance, glucose metabolism, immune escape, and metastasis [75,77], resulting in the reduced overall survival of patients in various cancers [75]. Consistently, the inhibition of HIF-1α can reduce the CSC numbers and suppress drug resistance in various cancer types, such as glioma, hematological cancers, and breast cancer [78-80].

EMT is widely known to induce stem-like properties in cancer cells [81]. The HIF-1 signaling pathway is crucial for the modulation and maintenance of CSCs and the EMT phenotype [82]. In thyroid and prostate cancer, HIF-1α-mediated EMT can increase stem-like cells [83,84]. In tumor tissues, the hypoxic or necrotic area of is considered a niche of CSCs. HIF-1 regulates CSC-signature genes, such as *CD44, CD133, OCT4, SOX-2, NANOG*, and *MYC*, that are increased in the CSCs of this niche. In pancreatic cancer, gastric cancer, and neuroblastoma, the discontinuous hypoxia upregulates HIF-1α, enhancing stem-like characteristics of theses cancer cells [85-87]. HIF-1 also plays an important role in promoting mammary tumor growth and metastasis by direct regulation of CSCs [87]. These studies highlight the vital role of HIF-1 in accelerating tumorigenesis, metastasis, and drug resistance because of CSC sustenance.

HIF-1α has been demonstrated to regulate PD-L1 expression on both tumor cells and myeloid-derived suppressor cells (MDSCs), leading to immune evasion [88]. HIF-1α also increases the secretion of vascular endothelial growth factor A (VEGEFA), thus promoting the recruitment of MDSCs and Tregs to the TME [89]. Furthermore, HIF-1α promotes the shedding of NKG2D ligands, causing tumor immune evasion from natural killer cells [90]. Owing to the complex regulatory network of HIF-1, designing specific and ideal inhibitors remains a challenge. Although several HIF-1α inhibitors have been studied and reported, so far none of them has been approved for clinical use [91]. Despite the incomplete success of direct HIF-1α antagonists, several other drugs, such as heat shock protein 90 (HSP90) inhibitors, are shown to have the potential to indirectly inhibit HIF-1α [92]. Anthracycline agents, including doxorubicin and daunorubicin can inhibit HIF-1α by suppressing the binding of HIF-1α to DNA [93]. Overall, given the role of HIF-1α in the immunosuppressive TME, HIF-1α inhibitors may hold promise for improving the efficiency of combined immunotherapy.

2.5 Hedgehog Signaling Regulates the PD-L1 Expression under Hypoxic Conditions

Hh is a conserved signaling pathway in the development of intercellular communication. Three ligands, including Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH) can activate Hh signaling [94]. The primary receptor for these ligands is Patched-1 (Ptch1). Without the ligand, Ptch1 suppresses smoothened (Smo), but upon the binding of ligand, Ptch1 inhibition is released and Smo is activated. Subsequently, Smo stimulates the glioma-associated oncogene (Gli) transcription factors Gli1, Gli2, and Gli3 [95]. Gli1 activates the target genes related to tumorigenesis as well as angiogenesis factor genes [96].

Hh signaling is aberrant in various types of cancers and contributes to cancer initiation, proliferation, progression, and invasion [97]. In pancreatic CSCs, SHH and other HH signaling components are expressed more than in normal pancreatic stem cells or pancreatic ductal epithelial cells [98]. In addition, Gli-independent Hedgehog signaling is observed in CSCs-enriched cancer and required for CSC survival. Thus, the dysfunction of HH signaling is considered one of the key events in CSCs origin.

Previous researches have demonstrated that Hh signaling promotes cell cycle-dependent tumor growth and invasion by improving the metalloproteinase expression [99,100]. Therefore, hedgehog inhibitors (HHIs) are used for therapy. However, HHIs do not meet the anticipated outcome. To clarify the cause, HH signaling itself should be considered, it is complex and plays a role not only in tumor development but also drug resistance. Of these, the mutation of signaling components is responsible for the non-effectiveness of HHIs. Interestingly, recent studies show that Hh signaling may modulate PD-L1 expression under hypoxic conditions. Additionally, Hh inactivation and/or the blockade of PD-L1 increases the anti-tumor activity of lymphocytes [101]. These results indicate that the action of Hh signaling may contribute to the ICIs resistance via PD-L1 expression and inhibition of the lymphocyte anti-tumor activity. The combination of ICIs and new generation HHIs in the future may shed insights into overcoming the development of resistance.

3. Summary

The different signaling pathways associated with CSCs may play a vital role in the immune resistance. The specific mechanisms inducing the immune resistance include—the recruitment of immunosuppressive cells, especially MDSCs and Treg cells, to the TME; enhancement of CSC properties, especially the EMT; the regulation of PD-L1

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expression on the tumor or CSC surface to inhibit CD8+ T cell cytotoxicity and even the direct loss of CD8+ T cells (Figure 2). Of note, hypoxia can directly induce PD-L1 expression in cancer cells; meanwhile, HIF-1α and HH signaling can be directly activated by hypoxia, thus contributing to the immune resistance. Moreover, these possible mechanisms may function together as a network rather than in isolation. However, to tackle the problem of immune resistance, considerable research efforts are needed to gain an accurate understanding of the underlying mechanisms.

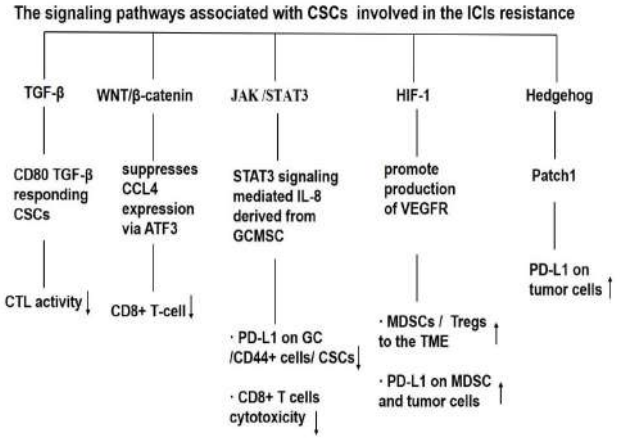

Figure 2. The Schematic Diagram for Signaling Pathways Associated with Cancer Stem Cells in Immunotherapy Resistance

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ARTICLE

Postoperative UFT-/Tegafur-based Chemotherapy Versus Postoperative Radiotherapy for Early-stage Non-small Cell Lung Cancer: A Systematic Review and Network Meta-analysis

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ABSTRACT

Background: Both of UFT-/Tegafur-based postoperative chemotherapy and postoperative radiotherapy have made large progress in treatment of early-stage non-small cell lung cancer. While it is unclear that, whether UFT-/Tegafur-based postoperative chemotherapy is superior to postoperative radiotherapy for early-stage non-small cell lung cancer with no direct evidence. **Methods:** Electronic databases (Pubmed, embase, cochrane library and clinicaltrials.gov) were searched to obtain relevant studies. This systematic review and meta-analysis is reported in accordance with the Preferred Items for Systematic Reviews and Meta-analysis (PRISMA) Statement and was registered at International Prospective Register of Systematic Reviews (number **CRD42018095979**). Sensitive analysis was conducted by excluding overweight studies. Funnel plot and egger's test were performed to conduct publication bias. **Results:** Twenty-one randomized control trials were included. Our results suggested UFT-/Tegafur-based postoperative chemotherapy could improve overall survival over postoperative radiotherapy [HR=0.69 (0.59-0.80), p=0.000]. But subgroup analysis about stage showed there was no significant difference between them, no matter of stage I, II and III. As to chemotherapy regime, both UFT-/Tegafur + platinum+vinca alkaloid [HR=0.68 (0.56-0.82), p=0.000] and UFT-/Tegafur only [HR=0.66 (0.54-0.79), p=0.000] were superior to radiotherapy. Subgroup analysis about radiotherapy delivery method and dose showed, significant improvement of chemotherapy over radiotherapy for Cobalt-60 only [HR=0.54 (0.39-0.75), p=0.000], Cobalt-60 and linac [HR=0.69 (0.59-0.81), p=0.000] and ≥45 Gy [HR=0.64 (0.54-0.75), p=0.000], but not for linac only [HR=0.78 (0.60-1.03), p=0.081] and ≥ 45 Gy [HR=0.86 (0.67-1.11), p=0.241]. **Conclusion:** UFT-/Tegafur-based postoperative chemotherapy was superior to postoperative radiotherapy for improving overall survival of early-stage non-small cell lung cancer, but it is not always so under certain circumstance, such as RT delivery method and radiation dose. Of course, it is imperative to further explore differences in specific stage, such as I A and I B.

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1. Introduction

Non-small cell lung cancer (NSCLC) is a malignant tumor with high mortality, accounting for about 85% of lung cancer. [1] Because of the high invasiveness and rapid progress, it is very important to carry out effective treatment of NSCLC in the early stage. Although surgical resection is currently the standard treatment for early NSCLC, long-term postoperative survival is unsatisfactory. [2-3] Therefore, many studies have explored the efficacy of postoperative UFT/Tegafur-based adjuvant chemotherapy and radiotherapy.

Through systematic retrieval, we have found that most studies have shown that UFT/Tegafur based adjuvant chemotherapy improves overall survival, [4-6] but postoperative radiotherapy seems not. [7-8] In addition, most clinicians also think that postoperative UFT/Tegafur-based adjuvant chemotherapy is better than postoperative radiotherapy, but there is no direct evidence. Moreover, new studies have found that postoperative radiotherapy may also improve survival rates in early non-small cell lung cancer patients. [9-10] Therefore, the difference of UFT/Tegafur-based postoperative adjuvant chemotherapy and postoperative radiotherapy in the treatment of early non-small cell lung cancer is puzzling. In recent years, network meta-analysis, a method of obtaining evidence from evidence-based medicine, has been paid much attention to. Indirect comparison, as a special type of meta-analysis with reliabie results, [11-12] is also widely used. [13-14] Given no report of direct comparison between UFT/Tegafur based postoperative adjuvant chemotherapy and radiotherapy in treatment of early-stage non-small cell lung cancer, we performed this systematic review and network meta-analysis, expecting to provide assistance for clinic.

2. Methods

2.1 Search Strategy

Relevant published or unpublished RCT studies were selected by searching Pubmed, Embase, Cochrane library and ClinicalTrials.gov. We used MESH terms “chemotherapy”, “radiotherapy”, “surgery” and “Carcinoma, non-small cell lung”, and the retrieval strategy of Pubmed as follow: surgery[Title/Abstract] OR “General Surgery” [Mesh] AND Therapy, Drug [Title/Abstract] OR Drug Therapies [Title/Abstract] OR Therapies, Drug [Title/Abstract] OR Chemotherapy [Title/Abstract] OR Chemotherapies Pharmacotherapy [Title/Abstract] OR Pharmacotherapies [Title/Abstract] OR “Drug Therapy” [Mesh] AND placebo [Title/Abstract] OR “Controlled Clinical Trial” [Publication Type] OR “Randomized Controlled Trial” [Publication Type] AND Carcinoma, Non Small Cell Lung [Title/Abstract] OR Carcinomas, Non-Small-Cell Lung [Title/Abstract] OR Lung Carcinoma, Non-Small-Cell [Title/Abstract] OR Lung Carcinomas, Non-Small-Cell [Title/Abstract] OR Non-Small-Cell Lung Carcinomas [Title/Abstract] OR Nonsmall Cell Lung Cancer [Title/Abstract] OR Non-Small-Cell Lung Carcinoma [Title/Abstract] OR Non Small Cell Lung Carcinoma [Title/Abstract] OR Carcinoma, Non-Small Cell Lung [Title/Abstract] OR Non-Small Cell Lung Cancer [Title/Abstract] OR “Carcinoma, Non-Small-Cell Lung” [Mesh] OR radiation therap* [Title/Abstract] OR PORT [Title/Abstract] OR Radiother* [Title/Abstract] OR “Radiotherapy” [Mesh] AND surgery [Title/Abstract] OR “General Surgery” [Mesh] AND Carcinoma, Non Small Cell Lung [Title/Abstract] OR Carcinomas, Non-Small-Cell Lung [Title/Abstract] OR Lung Carcinoma, Non-Small-Cell [Title/Abstract] OR Lung Carcinomas, Non-Small-Cell [Title/Abstract] OR Non-Small-Cell Lung Carcinomas [Title/Abstract] OR Nonsmall Cell Lung Cancer [Title/Abstract] OR Non-Small-Cell Lung Carcinoma [Title/Abstract] OR Non Small Cell Lung Carcinoma [Title/Abstract] OR Carcinoma, Non-Small Cell Lung [Title/Abstract] OR Non-Small Cell Lung Cancer [Title/Abstract] OR “Carcinoma, Non-Small-Cell Lung” [Mesh] AND placebo [Title/Abstract]) OR “Controlled Clinical Trial” [Publication Type] OR “Randomized Controlled Trial” [Publication Type]. Additional new studies were identified by reading included studies and relevant reviews. All of the postoperative chemotherapy regime was UTF/Tegarfur-based. This systematic review and meta-analysis is reported in accordance with the Preferred Items for Systematic Reviews and Meta-analysis (PRISMA) Statement and was registered at International Prospective Register of Systematic Reviews (number CRD42018095979). Randomized control trials were included if they met following criteria: (1) postoperative chemotherapy vs surgery alone; (2) postoperative radiotherapy vs surgery alone; (3) early-stage non-small cell lung cancer; (4) providing estimates of overall survival.

2.2 Data Extraction

Two authors (LX Yu and M Song) independently extracted the original data. Disagreement was resolved by discussion. The extracted data were consisted of the follow items: the first author’s name, publication year, methods, study design, matching criteria, total number of cases and controls, stage and therapy regime.

2.3 Statistical Analysis

Review manager 5.3 and Stata 14.0 were performed to conduct this meta-analysis. Taking low heterogeneity into

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account, we use fixed effect model to pool estimates. In addition, we excluded the researches with overweight to conduct sensitive analysis and implement subgroup analysis to explore the differences of postoperative chemotherapy and postoperative radiotherapy of non-small cell lung stage and therapy regime. Publication bias was tested by funnel plot and egger's test, and P value of egger's test < 0.05 is considered significant. Hazard ratio with 95%CI and odds ratio with 95%CI were used to assess estimates of survival.

3. Results

3.1 Eligible Studies

As shown in Figure 1, total twenty-one randomized control trials [15-35] were identified finally, eleven about postoperative UFT/Tegafur-based chemotherapy [15-25] and ten about postoperative radiotherapy. [26-35] Two studies were from Study Group for Adjuvant Chemotherapy for Lung Cancer (SGACLC ACTLC), and one study was from Lung Cancer Study Group (LCSG). Especially, one study obtained from the reference is an unpublished data. Characteristics of included studies were shown in Table 1. The range of size was from 58 to 999, and chemotherapy regime mainly contained UFT/Tegarfur + platinum + vinca alkaloid and UFT/Tegarfur only. Characteristics of included studies were shown in Table 1. Methodological quality graph and summary were in Figure 2 and Figure 3.

Table 1. Characteristics of included studies

Study, year	Methods	Size (n)	Intervention	Stage	Therapy regime
SGACLC ACTLC, 1992	RCT:1982 to 1985	306	Postoperative CT	NK	Cisplatin,mitomycin,tegafur
SGACLC ACTLC, 1995	RCT:1985 to 1987	332	Postoperative CT	Ⅰ，Ⅱ，Ⅲ	Cisplatin,doxorubicin,UFT
Wada H, 1996	RCT:1985 to 1988	208	Postoperative CT	Ⅰ，Ⅱ，Ⅲ	Tegarfur,uracil
	RCT:1985 to 1988	323	Postoperative CT	Ⅰ，Ⅱ，Ⅲ	Cisplatin,vindesine,UFT
Wada H, 1999	RCT:1988 to 1989	225	Postoperative CT	Ⅰ，Ⅱ	Cisplatin,vindesine,mitomycin,tegarfur,uracil
Xu G, 1998	RCT:1989 to 1992	70	Postoperative CT	Ⅰ，Ⅱ，Ⅲ	Cisplatin,vindesine,doxorubicin,cyclophosphamide
Imaizumi M, 2005	RCT:1982 to 1988	104	Postoperative CT	Ⅰ	Cisplatin,vindesine,tegarfur,uracil
	RCT:1992 to 1995	104	Postoperative CT	Ⅰ	Tegarfur,uracil
Nakagawa M, 2005	RCT:1991 to 1994	367	Postoperative CT	Ⅰ，Ⅱ	Tegarfur,uracil
Nakagawa K, 2006	RCT:1992 to 1994	172	Postoperative CT	Ⅰ	Tegarfur,uracil
	RCT:1992 to 1994	95	Postoperative CT	Ⅱ，Ⅲ	Cisplatin,vindesine,tegarfur,uracil
Sawamura K, 1988	RCT:1982 to 1987	321	Postoperative CT	Ⅰ	Tegarfur
	RCT:1982 to 1986	83	Postoperative CT	Ⅱ，Ⅲ	Doxorubicin,mitomycin,tegarfur
	RCT:1982 to 1987	28	Postoperative CT	Ⅱ	Cisplatin,tegarfur
Endo C, 2003	RCT:1992 to 1994	219	Postoperative CT	Ⅰ，Ⅱ	Tegarfur,uracil
Kato H, 2004	RCT:1994 to 1997	999	Postoperative CT	Ⅰ	Tegarfur,uracil
Chang Y,2015	Pooled analysis of RCT	58	Postoperative RT	Ⅰ	54 Gy in three 18 Gy fractions/ 50 Gy in four 12.5 Gy fractions within 5 days
					54 Gy in three 18 Gy fractions over 5-8 days/ 60 Gy in four 12 Gy fractions over 10-14 days
Park JH, 2007	RCT:1989 to 1998	111	Postoperative RT	Ⅱ，Ⅲ	50.4 to 55.8 Gy in 1.8 to 2 Gy fractions, 5 times a week
EORTC 0886, 2000	RCT:1986 to 1990	106	Postoperative RT	Ⅱ，Ⅲ	56 Gy in 28 fractions in 5.5 weeks
van Houtte P, 1980	RCT:1966 to 1977	224	Postoperative RT	Ⅰ，Ⅱ，Ⅲ	60 Gy in 30 fractions in 6 weeks
Feng QF, 2000	RCT:1981 to 1995	317	Postoperative RT	Ⅱ，Ⅲ	60 Gy in 30 fractions in 6 weeks
Dautzenberg B, 1999	RCT:1986 to 1994	189	Postoperative RT	Ⅰ，Ⅱ，Ⅲ	60 Gy in 24 to 30 fractions in 6 weeks
	RCT:1988 to 1994	539	Postoperative RT	Ⅰ，Ⅱ，Ⅲ	60 Gy in 24 to 30 fractions in 6 weeks
LCSG, 1986	RCT:1978 to 1985	230	Postoperative RT	Ⅱ，Ⅲ	50 Gy in 25 to 27.5 fractions in 5 to 5.5 weeks
Stephens RJ, 1996	RCT:1986 to 1993	308	Postoperative RT	Ⅱ，Ⅲ	40 Gy in 15 fractions in 3 weeks
Lafitle JJ, 1996	RCT:1985 to 1991	163	Postoperative RT	Ⅰ	45 to 60 Gy in 22.5 to 30 fractions in 6weeks
Trodella L, 2002	RCT:1989 to 1997	104	Postoperative RT	Ⅰ	50.4 Gy in 1.8 Gy/d in 5 weeks and 3 days

NK, not known; RCT, randomised controlled trial; CT, chemotherapy; RT, radiotherapy; Gy-Gray,unit of radiotherapy dose; UFT, Uracil/tegafur

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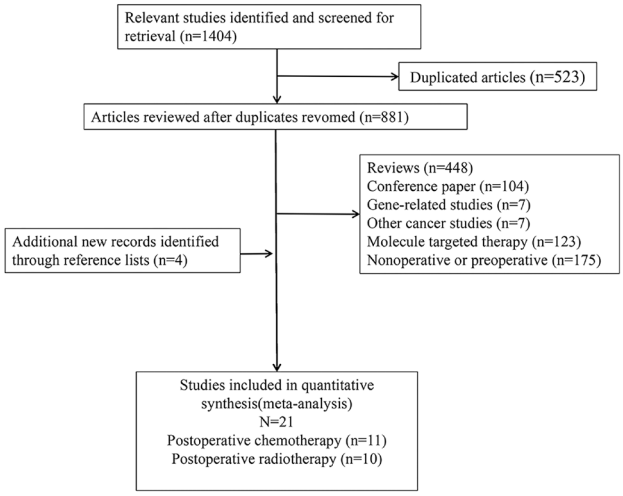

Figure 1. Quality of reporting of meta-analyses flow diagram.

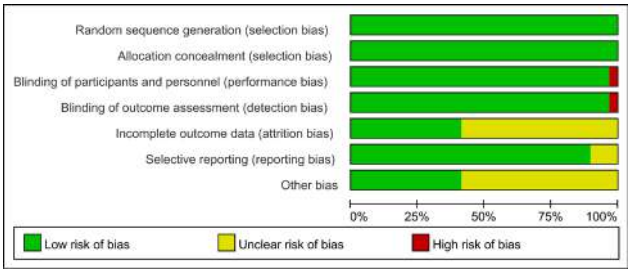

Figure 2. Methodological quality graph: review authors' judgements about each methodological quality item presented as percentages across all included studies

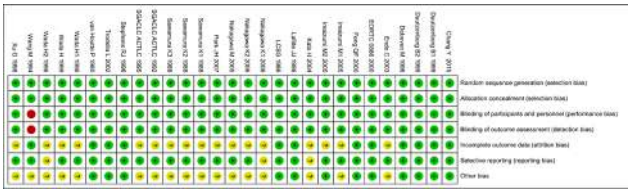

Figure 3. Methodological quality summary: review authors' judgements about each methodological quality item for each included study

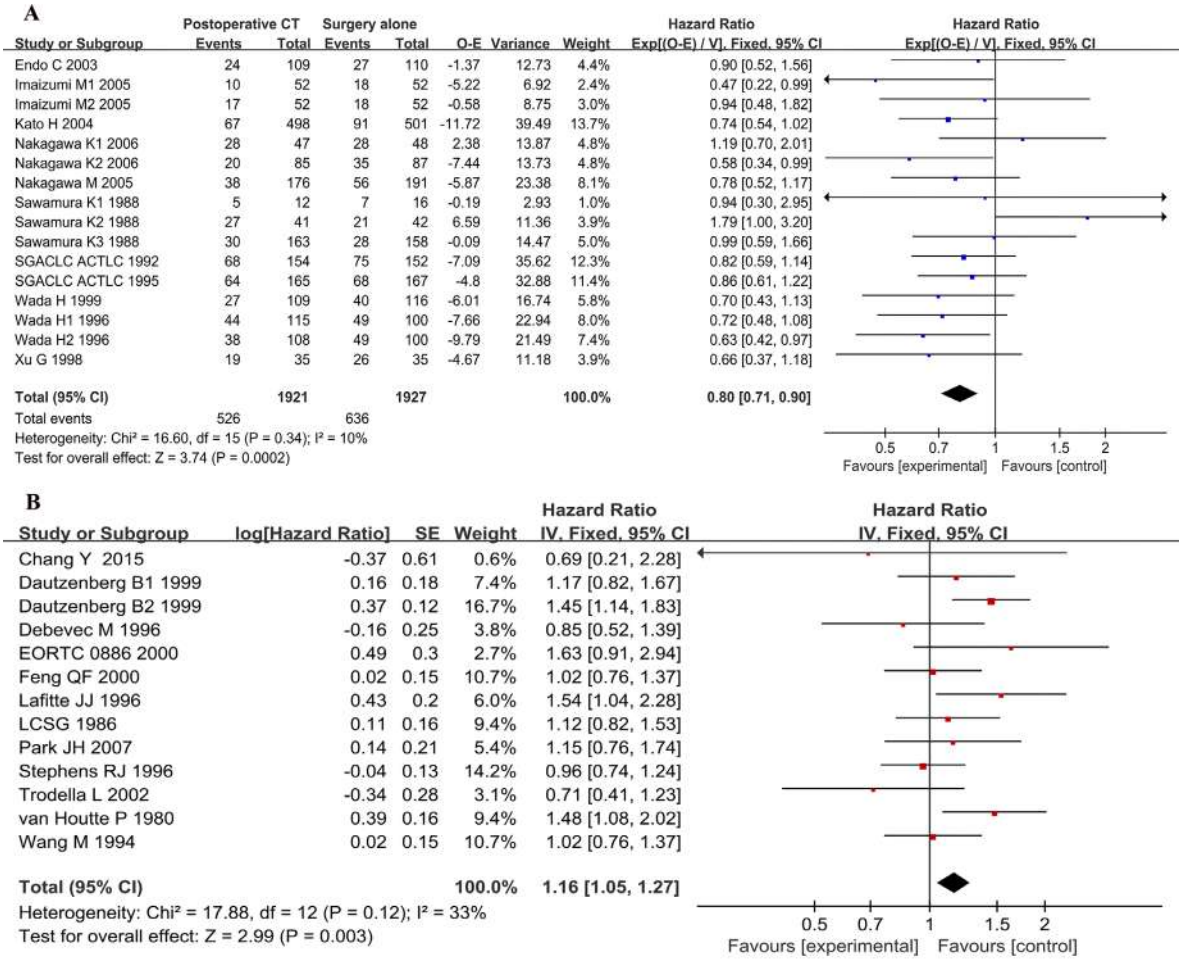

Figure 4. Forest plots of postoperative chemotherapy vs surgery alone group (A) and postoperative radiotherapy vs surgery alone group (B)

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3.2 Overall Survival

For overall survival, the pooled Hazard Ratios of death were 0.80 (0.71-0.90, p=0.0002) and 1.16 (1.06-1.27, p=0.003) in postoperative UFT/Tegarfur-based chemotherapy vs surgery alone group and postoperative radiotherapy vs surgery alone group, respectively. Network indirect comparison suggested that postoperative UFT/Tegarfur-based chemotherapy could improve overall survival over postoperative radiotherapy [HR=0.69 (0.59-0.80), p=0.000], which was shown in Table 2.

3.3 Subgroup Analysis

To explore potential influential factors, subgroups analysis about non-small cell lung cancer stage and therapy regime were performed. For stage, there no evidence of important statistical significance between postoperative chemotherapy and postoperative radiotherapy [stage Ⅰ HR=0.80 (0.64-1.00), p=0.051, stage Ⅱ HR=0.79 (0.50-1.26), p=0.324, stage Ⅲ HR=0.88 (0.58-1.36), p=0.574]. For chemotherapy regime, both UFT/Tegarfur+platinum+vinca alkaloid and UFT/Tegarfur only could improve overall survival over radiotherapy [HR=0.68 (0.56-0.82), p=0.000, 0.66 (0.54-0.79), p=0.000]. In terms of RT delivery method, postoperative chemotherapy is superior to postoperative radiotherapy in Cobalt-60 only [HR=0.54 (0.39-0.75), p=0.000] and Cobalt-60 and linac [HR=0.69 (0.59-0.81), p=0.000], but not in linac only[HR=0.78 (0.60-1.03), p=0.081]. Similarly, with ≥45 Gy radiation dose, there existed significant difference between postoperative chemotherapy and postoperative radiotherapy [OR=0.64 (0.54-0.75), p=0.000], while not with ＜ 45 Gy radiation dose [OR=0.86 (0.67-1.11), p=0.241]. The main results were shown in Table 2.

Table 2. Summary effect of survival index

Outcome/Subgroup	No. Of patients	Statistical method	Effect size (relative value)	P value
Overall survival	3956/2349	Hazard Ratio (Fixed, 95%CI)	**0.69 (0.59-0.80)**	**0.000**
Subgroup (stage)				
Stage I	2574/572	Hazard Ratio (Fixed, 95%CI)	0.80 (0.64-1.00)	0.051
Stage II	190/817	Hazard Ratio (Fixed, 95%CI)	0.79 (0.50-1.26)	0.324
Stage III	178/746	Hazard Ratio (Fixed, 95%CI)	0.88 (0.58-1.36)	0.574
Subgroup (chemotherapy regime)				
UFT/Tegafur+P+VA	1375/2349	Hazard Ratio (Fixed, 95%CI)	**0.68 (0.56-0.82)**	**0.000**
UFT/Tegafur only	2390/2349	Hazard Ratio (Fixed, 95%CI)	**0.66 (0.54-0.79)**	**0.000**
Subgroup (RT delivery method)				
Cobalt-60 only	3956/202	Hazard Ratio (Fixed, 95%CI)	**0.54 (0.39-0.75)**	**0.000**
Cobalt-60 and linac	3956/2063	Hazard Ratio (Fixed, 95%CI)	**0.69 (0.59-0.81)**	**0.000**
Linac only	3956/395	Hazard Ratio (Fixed, 95%CI)	0.78 (0.60-1.03)	0.081
Subgroup (radiation dose)				
≥45 Gy	3956/2019	Odds Ratio (Fixed, 95%CI)	**0.64 (0.54-0.75)**	**0.000**
＜ 45 Gy	3956/382	Odds Ratio (Fixed, 95%CI)	0.86 (0.67-1.11)	0.241
No. Of patients, postoperative chemotherapy/postoperative radiotherapy **P+VA, platinum+vinca alkaloid**				

3.4 Sensitive Analysis and Publication Bias

We excluded overweight studies, such as Kato et al, SGACLC ACTLC and Dautzenberg2 et al, to conduct sensitive analysis, and final result was not changed [HR=0.69 (0.57-0.84), p=0.000]. Funnel plots were shown in Figure 4. Egger's test suggested that there was no publication bias in postoperative UFT/Tegarfur-based chemotherapy group (p=0.637) and postoperative radiotherapy group (p=0.417).

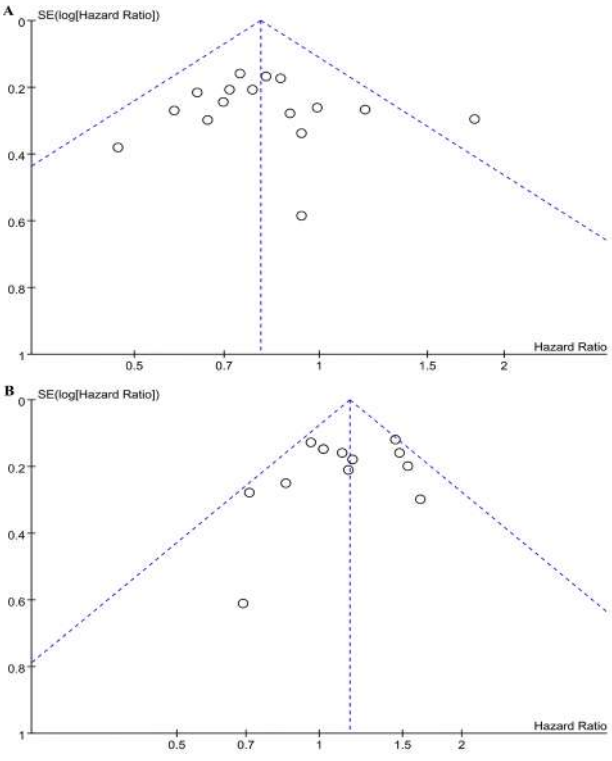

Figure 5. Funnel plots of postoperative chemotherapy vs surgery alone group (A) and postoperative radiotherapy vs surgery alone group (B)

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4. Discussion

Surgical resection is the recommended method for the treatment of non-small cell lung cancer, but the postoperative survival rate is always unsatisfactory, even in the early stage, the 5-year survival rate is only 45.1%, [36] so the choice of postoperative adjuvant treatment is very important. Recent years, many scholars have studied the effects of postoperative UFT/Tegarfur-based adjuvant chemotherapy and adjuvant radiotherapy in the treatment of early-stage non-small cell lung cancer. The results showed that UFT/Tegarfur-based adjuvant chemotherapy seemed to be superior to postoperative adjuvant radiotherapy, but there was no definitive comparative evidence. Therefore, we wonder much that UFT / Tegarfur based adjuvant chemotherapy is really better than postoperative adjuvant radiotherapy? If so, is it true for every aspect, such as specific stage? Based on that, we conducted the network meta-analysis. Our results showed that UFT/Tegarfur based adjuvant chemotherapy could significantly improve the overall survival rate of patients [HR=0.69 (0.59-0.80) p=0.000] compared with postoperative adjuvant radiotherapy, but it also changed with different stages and radiotherapy methods.

UFT is an oral fluorouracil preparation that combines tegafur, a prodrug of 5-fluorouracil, with uracil, which inhibits dihydropyrimidine dehydrogenase, the rate-limiting enzyme responsible for 5-fluorouracil catabolism. Tegafur, the major component of UFT, is metabolized to gamma-hydroxybutyric acid and gammabutyrolactone, which inhibit angiogenesis. In recent years, UFT/Tegarfur-based postoperative adjuvant chemotherapy has made great progress in the treatment of early non-small cell lung cancer. Hotta K et al [4] discovered that therapy with tegafur and uracil (UFT; HR, 0.799; 95% CI, 0.668 to 0.957; P =0.015) could yield a significant survival benefit to early-stage NSCLC. In 2005, Hamada C et al [37] showed that postoperative adjuvant chemotherapy with UFT was associated with improved 5- and 7-year survival in a Japanese early-stage NSCLC patient population, whose overall pooled hazard ratio was 0.74 and 95% CI was 0.61 to 0.88 (P =0.001). And in 2009, Hamada C et al [6] reported significant hazard ratio even was 0.62, with much better than before. UFT/Tegarfur based postoperative adjuvant chemotherapy may be promising for early-stage NSCLC.

Most previous studies [7-8] have shown that postoperative radiotherapy couldn't effectively improve the survival rate of early non-small cell lung cancer patients, so the clinical treatment of this program is relatively conservative. But the latest researches have come to the opposite conclusions. Sakib N et al [9] suggested that the addition of PORT significantly improves survival in patients with resectable stage IIIA-N2 NSCLC [HR=0.73（0.58-0.92）,P = 0.008]. Likewise, Patel SH et al [10] reached similar conclusion in III-N2 NSCLC [HR=0.73 （0.58-0.92）,P = 0.008]. In the face of this outcome, we included randomized controlled trials of higher quality, and the results suggested that postoperative radiotherapy might not improve the survival rate of patients with early non-small cell lung cancer [HR = 1.16 (1.06-1.27), P = 0.003]. But this does not necessarily mean that UFT/Tegarfur-based postoperative adjuvant chemotherapy is superior to postoperative radiotherapy in all aspects.

We therefore further compared the effects of UFT/Tegarfur-based postoperative adjuvant chemotherapy with postoperative radiotherapy, and performed a comprehensive analysis of the different stages, chemotherapy regimens, radiotherapy methods and doses of the subgroups Our results suggest that UFT/Tegarfur-based postoperative adjuvant chemotherapy does improve survival in patients with early-stage non-small cell lung cancer [HR = 0.69 (0.59-0.80), P = 0.000], regardless of the chemotherapy regimen（Table 2）. [UFT/Tegafur+P+VA, HR= 0.68 (0.56-0.82), p=0.000; UFT/Tegafur only, HR= 0.66 (0.54-0.79), p=0.000]. However, no significant difference exhibited in stage. [Stage Ⅰ, HR= 0.80 (0.64-1.00), p=0.051; Stage Ⅱ, HR= 0.79 (0.50-1.26), p=0.324; Stage Ⅲ, HR= 0.88 (0.58-1.36), p=0.574] （Table 2）. We may also need sufficient data to further refine staging studies, such as I A, I B, II A, III A. In terms of radiotherapy methods and doses, the results are inconsistent. In the cobalt-60, Cobalt-60 + linac and≥45Gy, the UFT/Tegarfur based postoperative adjuvant chemotherapy could improve early-stage NSCLC overall survival over postoperative radiotherapy [Cobalt-60 only, HR=0.54 (0.39-0.75), p= 0.000; Cobalt-60 and linac, HR= 0.69 (0.59-0.81), p= 0.000; ≥45 Gy, HR= 0.64 (0.54-0.75), p= 0.000]（Table 2）, However, when Linac only and < 45 Gy, there was no significant difference between the two adjuvant regimens. [Linac only, HR= 0.78 (0.60-1.03), p= 0.081; < 45 Gy, HR= 0.86 (0.67-1.11), p= 0.241].（Table 2）.Therefore, UFT/Tegarfur-based postoperative adjuvant chemotherapy isn't always superior to radiotherapy, and the reasons need to be further explored. Sensitivity analysis and publication bias test showed that our results were stable and reliable.

We also need to point out the limitations of our research. First, we do not have enough data for more detailed phased studies, which may be an important reason for the differences in outcomes. Secondly, whether there are differences in the effectiveness of histology is the question we will explore in the future. Finally, we failed to match sample size completely.

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5. Conclusion

Our study suggests that UFT/Tegarfur based postoperative adjuvant chemotherapy may not always be superior to postoperative radiotherapy, and it seems to be closely related to specific treatment methods, especially different radiotherapy interventions. Of course, detailed stage needs to be explored in the future. Our results change our previous understanding that postoperative UFT/Tegarfur-based chemotherapy is always superior to postoperative radiotherapy, which allows us to weigh the options of different methods.

List of abbreviations

Randomized control trials, RCT; Non-small cell lung cancer, NSCLC; Study Group for Adjuvant Chemotherapy for Lung Cancer, SGACLC ACTLC; Lung Cancer Study Group, LCSG; Hazard RatioHR.

Declarations

Ethical Approval and Consent to participate: Non-essential Consent for publication: All authors agree. Availability of data and material: All data and material are Availabile. Competing interests: The authors report no conflicts of interest in this work Funding: None.

Authors' Contributions

LX Yu and M Song conceived and designed the methods, extracted the original data and drafted the manuscript. LX Yu and SF Ji performed statistical analysis. SF Ji interpreted results and revised the manuscript. SF Ji and M Song had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of data analysis.

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REVIEW

Schistosomal Colorectal Cancer: Biomarkers and Treatment Strategies

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ABSTRACT

About 15.4% of human cancers worldwide have been attributed to infections. Among these, blood and liver flukes, notably *Schistosoma* sp, *Clonorchis Sinesis*, and *Opisthorchis Viverrini* have been associated with the development of various cancer types. *Schistosoma* sp. promotes colorectal cancer (CRC) progression through multiple mechanisms including production of toxins, symbiotic action with bacterial agents, and more importantly chronic inflammation. Diagnosis of schistosomal colorectal cancer (SCC) requires high index of clinical suspicion in endemic areas. Novel biomarkers may aid early diagnosis of SCC in patients with chronic intestinal schistosomiasis. Treatment should be tailored to individual patients according to the stage and biologic characteristics of the tumour, and the extent of hepatosplenic schistosomiasis. Long-term survival after surgical resection of SCC is lower than that reported in patients with sporadic CRC.

1. Introduction

Colorectal cancer (CRC) is the third most common human cancer. Accounting for approximately 1.8 million new cases and 861,000 deaths in 2018, it was considered the second leading cause of cancer death worldwide. [1] In addition to genetic factors, several environmental influences may interplay in a complex multistep process to promote colorectal carcinogenesis. These include cigarette smoking, high alcohol consumption, obesity, lifestyles, and oncogenic viral and bacterial agents. [2-4] Recently, we highlighted the role of *Schistosoma* sp., a digenetic blood fluke, on the aetiology of colorectal cancer, disease progression and the characteristics of patients. [5,6]

*Schistosoma*l colorectal cancer (SCC) has been linked to *S. japonicum* and *S. mansoni*, the leading causative agents of intestinal schistosomiasis, and it has been mainly reported in areas of high endemicity of schistosomal infection; Southeast Asia, Africa, and the Middle East. [5] The disease occurs in younger age group with male to female ratio being consistently higher than sporadic colorectal cancer. [7-9] Moreover, SCC exhibits more aggressive biological behaviour with a larger tumour size at presentation, frequent multifocal and multi-centric distribution, and mucinous histology. [7,10-13]

The therapeutic landscape of CRC has evolved significantly in recent years. Current and emerging treatment options include surgical resection, chemoradiation, biologic therapy, and immunomodulation.[14] Recent research works keep insight into predictive and prognostic biomarkers of CRC, which may aid diagnosis and the development of new treatment strategies. In the current review, we discuss

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the pathogenesis, diagnosis, and treatment options of SCC pointing to novel biomarkers and potential therapeutic targets in context.

2. Pathogenesis

The underlying pathogenesis of SCC involves several mechanisms, with chronic inflammation seems to play a pivotal role (Figure 1). These include production of schistosomal toxins notably schistosome worm antigen (SWA), soluble egg antigen (SEA), and inducible nitric oxide synthase (iNOS), the presence of endogenously produced carcinogens such as reactive nitrogen and oxygen species, down-regulation of immune surveillance, thereby favouring tumour progression and conferring a survival advantage to *Enterobacteriaceae* infections, particularly *Salmonella* sp. [6] The latter, in turn, promotes tumorogenesis directly through multiple epigenetic mechanisms, or indirectly through activation of environmental carcinogens. [15-17]

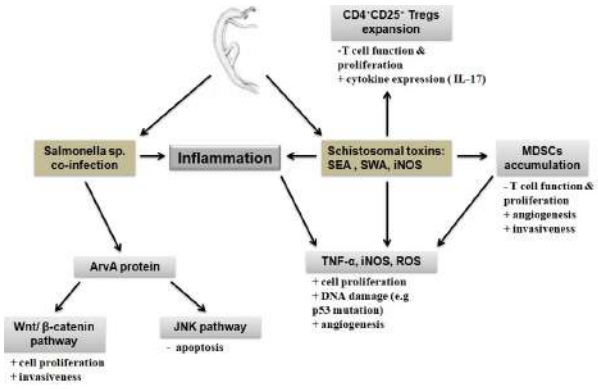

Figure 1. Illustration of the possible mechanisms of schistosome-induced colorectal carcinogenesis

3. Molecular Biomarkers

Several molecular changes have been described with SCC. Zhang et al. observed a different mutation types in the p53 gene, and a marginally significant higher proportion of base-pair substitutions at CpG dinucleotides and arginine missense mutations in the p53 gene among *S. japonicum*-associated rectal cancer patients compared to those with ordinary rectal cancer. [18] For *S. mansoni*-associated CRC, it was shown that schistosomal infection is associated with microsatellite instability, which is a sign of defective DNA repair. [19,20] This genomic instability results in DNA replication errors that preferentially affect target genes such as transforming growth factor (*TGH*) *bRII* and insulin-like growth factor (*IGF*)*2R*, and render them incapable of normal colonocytes homeostasis resulting in malignant growth. [21] Madbouly et al. evaluated the expression of p53 in patients with SCC, and found that mutant p53 overexpression was significantly more frequent in schistosomal than in non-schistosomal colorectal cancer. Moreover, p53 overexpression in SCC correlated well with nodal metastasis, mucinous carcinoma, and tumour multicentricity, thereby serving as a useful prognostic biomarker. [22] Zalata and his associates developed a more comprehensive study of the expression pattern of p53, Bcl-2, and C-Myc in 75 CRC cases; 24 of these had pathological evidence of *S. mansoni* infection. Although they did not find a significant association between parasitism and p53 and C-Myc expression, their results showed that SCC are characterized by Bcl-2 overexpression and less apoptotic activity than ordinary colorectal tumours. [23]

4. Diagnosis

The clinical presentation of SCC is often non-specific with common gastrointestinal symptoms such as altered bowel habits and rectal bleeding which could be attributed to chronic schistosomiasis or other gastrointestinal diseases. [7,24] Therefore, in non-endemic areas, the diagnosis requires high index of clinical suspicion in patients with history of schistosomal infection. Recent reports have evidenced that *S. mansoni*-associated CRC is associated with significantly higher serum levels of Telomerase, LDH, clusterin protein, and CEA when compared to intestinal *S. mansoni* infection only. [25,26] These biomarkers might serve as promising tools for early tumour detection in patients with chronic intestinal schistosomiasis.

Current methods of investigation of SCC involve colonoscopy and computed tomographic (CT) scanning, whereas histological analysis remains the gold standard to confirm the diagnosis. Colonoscopy not uncommonly reveals features of concomitant colonic schistosomiasis; acute colitis, chronic colitis, or mixed-type colitis, with presence of typical yellow nodules in the majority of these cases. [7,27] The endoscopic appearance of SCC is heterogeneous, but the most prevalent findings are ulcerative and fungating masses in the colonic wall, which are not uncommonly multi-focal. [7,10,11] Histology frequently reveals mucinous adenocarcinomas, with deposited ova in the tumour or the adjacent lamina propria (Figure 2). [12,13] Enhanced CT scan and virtual CT colonography were both shown to be highly valuable tools in the detection, characterization, and management of the SACC. The intestinal wall appears irregularly thickened in all patients, involving a wide range of the intestine. Other common CT features include spotty and patchy calcifications with obscured margins, tram-track calcifications and soft tissue

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masses. [10,11]

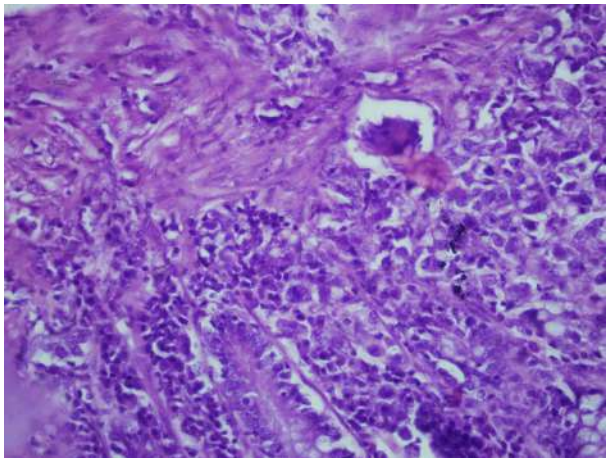

Figure 2. Photomicrograph showing *S. mansoni* egg shell inside the tumour and dysplastic glands. H&E × 40

5. Treatment and Outcome

5.1 Surgical

Complete mesorectal excision (CME) remains the best treatment modality for localized colon cancer that is amenable for curative surgical resection (70-80%), and provides effective palliation for metastatic disease. [28] For rectal cancer, curative surgery options are trans-anal and trans-sphincteric local excision, and total mesorectal excision (TME) with or without sphincter preservation. Laparoscopic-assisted approach is preferred over open colorectal resection, and confers better short-term outcomes. [29] In a series of 280 patients with SCC, 87 patients had laparoscopic resection, and 193 had open surgery. The laparoscopic group had earlier postoperative recovery, shorter hospital stay, and less surgical morbidities, with no increase in intra-operative adverse events. Higher rates of schistosomiasis-related complications were noted among the open surgery group. It was concluded that laparoscopic treatment is safe and effective for SCC with Child-Pugh grade A and B. [30] These results were recently replicated in CRC patients with liver cirrhosis caused by various infectious and non-infectious aetiologies. [31]

Generally, patients with SCC have significantly lower disease-free and overall survival than those with sporadic CRC. [13,32] These observations could be ascribed to the aggressive biological behaviour of SCC and to the presence of concomitant hepatosplenic schistosomiasis. Furthermore, the pattern of *Schistosoma* eggs deposition correlates well with the overall survival, but it does not affect the risk of anastomotic leak, indicating that the current standard surgical resection of SCC appears to be sufficient. [33]

5.2 Non-surgical

Preoperative (neoadjuvant) 5-Fluorouracil (5-FU) based chemoradiation or short-course high dose radiation therapy is currently the standard of care for operable T3/4 or node-positive rectal cancer.[34] Following surgery for CRC, various regimens of adjuvant treatment are used to achieve local control and to prevent systemic tumour dissemination, among which combination chemotherapy with oxaliplatin has the best curative effect and gives most benefit to patients.[35] For metastatic disease, oxaliplatin-based (FOLFOX) and irinotecan-based (FOLFIRI) regimens are regarded as first-line chemotherapy with comparable efficacy and overall survival. [29] The former regimen is particularly beneficial in treatment of SCC which frequently expresses high levels of clusterin. [26] Nonetheless, as hepatic perilobular and periportal fibrosis, leading to portal blood flow obstruction are frequent pathological findings, and active HBV/HCV coinfection is not uncommonly seen in patients with schistosomal infection regardless of the development of colonic schistosomiasis, [36,37] the use of oxaliplatin-based chemotherapy should be cautiously considered in SCC patients with portal hypertension, even in those with good liver reserve. This is because of the risk of hepatotoxicity, sinusoidal obstruction syndrome, and subsequent upper gastrointestinal bleeding. Oxaliplatin-based regimen is furthermore associated with a significantly increased mortality in portal hypertension patients undergoing colorectal cancer surgery. [38,39] Other conventional (NON-OXALI) chemotherapeutic regimens can be acceptable alternatives in those patients.

In the last decade, novel biologic therapies, targeting either epidermal growth factor signalling or angiogenesis, have been used in combination with cytotoxic agents as standard regimens for metastatic and advanced CRC. Inhibitors of the vascular endothelial growth factor (VEGF), an angiogenic molecule expressed in many patients with CRC and preferentially over-expressed on SCC cells, [11] among others, were shown to improve the progression-free and overall survival in advanced CRC with variable efficacy depending on the concurrent chemotherapy regimen utilized. [29] More recently, immunotherapy has emerged as a promising therapeutic option that selectively targets cancer-dependent pathways and avoids chemotherapy-related toxicities, thereby improves patient tolerance. This modality comprises immune checkpoints modulation, adoptive cell transfer, cancer vaccines, and oncolytic viral therapy. Nevertheless, the immunomodulating agents that have been investigated so far showed either minimal efficacy or have not yet proceeded on to later phase studies. [40] The clinical response rate to immunothera-

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py and progression-free survival could be significantly ameliorated by targeting certain subsets of CRC such as mismatch repair-deficient (MMR-d) and microsatellite instability-high (MSI-H) metastatic tumours, which account for approximately 15% of all CRC and 42% of SCC. [40,41] Additionally, modulating MDSC- and Treg-mediated immunosuppression may be a beneficial strategy to improve the efficiency of immunotherapeutic interventions, particularly in SCC cases. [42,43]

6. Conclusion

Although the carcinogenesis induced by *Schistosoma* sp. has been actively investigated, the causal relationship between the parasite and CRC is still poorly understood. The molecular biology of SCC must be further studied. Identification of predictive and prognostic biomarkers at an early stage is of paramount importance if the long-term outcome of surgery is to be improved. Further studies are warranted to explore new treatment strategies for SCC, and more effective means of controlling schistosomiasis in endemic areas.

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ARTICLE

Polymerase-tautomeric Model for Untargeted Delayed Base Substitution Mutations Formation during Error-prone and SOS Replication of Double-stranded DNA Containing Thymine and Adenine in Some Rare Tautomeric Forms

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ABSTRACT

Polymerase-tautomeric model for untargeted delayed base substitution mutations is proposed. Structural analysis of bases insertion showed that any canonical bases may be inserted opposite rare tautomeric forms of thymine T_3*, adenines A_2* and A_4* so that between them hydrogen bonds are formed. Canonical adenine and cytosine can be incorporated opposite canonical thymine only. Canonical thymine and guanine can be incorporated opposite canonical adenine only. If in the synthesis of DNA containing rare tautomeric forms of thymine T_3*, adenines A_2* and A_4*, involved DNA polymerases with relatively high fidelity of synthesis, mutations not appear. However, if further DNA synthesis will involve DNA polymerases having a low fidelity of synthesis, there may be base substitution mutations. It was shown that the conclusion made in the Tomasetti and Vogelstein cancer risk model that the formation of about 67% of all mutations was not caused by exposure to any mutagens is erroneous.

1. Introduction

The mutations formation is the main cause of cancer [1,2]. Untargeted delayed base substitution mutations are delayed base substitution mutations are formed on so-called undamaged DNA sites. They are part of radiation-induced genomic instability [3]. Radiation-induced genomic instability result in radiation-induced cancer [4,5]. Untargeted mutations are mutations that appear on the so-called undamaged sites of DNA [6-16]. Untargeted and untargeted delayed mutations are considered as radiation-induced bystander effects [17-20]. The generally accepted polymerase paradigm assumes that opposite DNA damage DNA polymerases incorporate bases that are unable to form hydrogen bonds with matrix bases [6,7,21–25].

Based on experimental facts, let's analyze polymerase paradigm of mutagenesis. An analysis of the work of various DNA polymerases showed [26] that specialized and modified DNA polymerases incorporate canonical

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bases opposite cyclobutane pyrimidine dimers, capable of forming hydrogen bonds with matrix bases. Several works [27–29], performed in recent years, were devoted to testing the tautomeric hypothesis of Watson and Crick [30]. In the active centers of DNA polymerases, noncanonical base pairs of guanine – thymine [27] and cytosine – adenine [28] were found, one of the bases in each pair being in a rare tautomeric form. Therefore, experiments show that always, even with error-prone or SOS DNA synthesis, complementary base pairing occurs, but one of the bases may be in a rare tautomeric form. The hypothesis that noncomplementary base pairing occurs is contrary to these experimental facts. Thus, within the framework of the generally accepted polymerase paradigm, it is impossible to explain the mechanisms of formation of targeted and untargeted base substitution mutations [26,31–33].

The Streisinger model [34] is used to explain frameshift mutations [35-37]. However, within the framework of the polymerase paradigm, it is absolutely not clear how *cis-syn* cyclobutane pyrimidine dimers can lead to frameshift mutations, and why, in some cases, they cause base substitution mutations, and in others, frameshift mutations. Within the framework of the polymerase paradigm, there is no complete understanding of the mechanisms of the targeted insertions [38,39], targeted deletions [40,41], and targeted complex mutations formation [42]. Several explanations have been proposed for radiation-induced bystander effects [3,20,43,44]. It is concluded that the nature and mechanisms of the formation of radiation-induced bystander effects are not fully understood [3,20,43,44]. The nature of delayed mutations are not known [3,43,45].

An analysis of the currently available models of mutagenesis shows that, within the framework of the generally accepted polymerase paradigm, it is not possible to exhaustively explain the mechanisms of the formation of any mutations. Therefore, to solve the problems of mutagenesis, a fundamentally different approach should be tried. In 1953, Watson and Crick suggested that mutagenesis may be based on the ability of DNA bases to be in various tautomeric forms [30]. In the future, this idea is being actively developed [46-51].

I have proposed and are developing polymerase-tautomeric models of targeted ultraviolet mutagenesis [26,31,33,38-42,52-57], radiation-induced bystander effects [58-62], and radiation-induced genomic instability [32,52,62-66]. I proposed a mechanism for rare tautomeric forms of DNA bases formation [33,68-70]. The formation of five rare tautomeric states of thymine and adenine [33,56,57] and seven of guanine and cytosine [31,71,72] is possible. DNA bases can form rare tautomeric forms as a result of the fact that hydrogen atoms between the bases can pass to their partners in hydrogen bonds [33,56,57]. They are also preserved during DNA synthesis - at the moment when such photodimers are in a single strand and therefore come into contact with water molecules for some time [33,57].

As shown by quantum chemical calculations, as a rule, hydrogen atoms return to their original position [73–77]. But opposite cyclobutane pyrimidine dimers, the DNA strand is bent and the hydrogen bonds between the bases that make up cyclobutane dimers or the bases adjacent to the photodimers are significantly weakened or are broken [78–82]. Therefore, hydrogen atoms between bases located in different DNA strands that formed pairs cannot return to their previous partners in hydrogen bonds, they will remain in new positions. This means that the tautomeric state has changed in these bases, and it will be stable [31,33,56,57,71,72]. To justify the polymerase-tautomeric models, K. B. Tolpygo and I performed several cycles of quantum-mechanical calculations devoted to studying the properties of excited hydrogen bonds in DNA [52,83–87].

I developed mechanisms for targeted base substitution mutations formation [26,32,33,52,55,66], targeted insertions [32,33,39,66], targeted deletions [32,33,40,41,66], delayed targeted base substitution mutations [63,64,66] and targeted complex insertions [32,33,42,66] under error-prone and SOS synthesis of DNA containing *cis-syn* cyclobutane thymine dimers. Mechanisms have been developed for formation of targeted base substitution mutations [31,54] and frameshift mutations (insertion) [38] with error-prone and SOS synthesis of DNA containing *cis-syn* cyclobutane cytosine dimers. In addition, a mechanism was proposed for formation of hot and cold spots of ultraviolet mutagenesis [53].

The formation of five *cis-syn* cyclobutane thymine dimers TT_1*, TT_2*, TT_3*, TT_4* and TT_5*, containing thymine molecules in rare tautomeric forms is possible [57]. *Cis-syn* cyclobutane thymine dimers TT_1*, TT_4* and TT_5* can cause only targeted base substitution mutations [26,32,33,66]. *Cis-syn* TT_2* cyclobutane thymine dimers can lead to targeted insertions [32,33,38,39,66] or targeted deletions only [32,33,40,41,66]. *Cis-syn* TT_3* cyclobutane thymine dimers can cause delayed targeted base substitution mutations only [63,64,66]. A DNA site containing *cis-syn* cyclobutane thymine dimers with thymine molecules in various tautomeric forms can lead to complex targeted mutations, for example, complex insertions [32,33,42].

I developed mechanisms for untargeted base substitution mutations formation [32,58–61,66] and untargeted

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insertions [32,62]. Their source is DNA bases in certain rare tautomeric forms located in small neighborhoods of cyclobutane dimers [58–62]. A detailed substantiation of the untargeted mutations is given in Ref. [61]. I developed a mechanism for the formation of targeted delayed base substitution mutations caused by *cis-syn* cyclobutane thymine dimers [32,52,63,64,66] and cytosine dimers [65].

Experimental studies in which noncanonical base pairs of guanine – thymine [27] and cytosine – adenine [28] with one of the bases in rare tautomeric forms were found in the active centers of DNA polymerases unambiguously demonstrate that tautomeric base pairs can form in active sites of polymerase [27,28]. This provides strong support for the ideas of Watson and Crick [30] and the polymerase-tautomeric models for mutagenesis through direct structural evidence [27,28].

Ultraviolet light induces delayed mutations [17,89]. The delayed mutations are usually point mutations [88], delayed mutations is usually not removed [90]. The genomic instability results in cancer [91]. The mechanism of delayed mutations formation is not clear [3,92-96]. Let us examine how untargeted delayed base substitution mutations can form.

2. Features of DNA Synthesis

DNA polymerases insert DNA bases opposite damaged DNA sites [97]. Translesion synthesis can cause mutations [99]. Mutations cab form as a result of the mechanism of the sliding clamp [100] or by the operation of low synthesis accuracy specialized DNA polymerases [7,101] (more detail see in Ref. [58]).

In order to understand how untargeted delayed base substitution mutations can be formed, we must understand how untargeted base substitution mutations are formed and how targeted delayed base substitution mutations are formed.

3. Polymerase-tautomeric Model for Untargeted Delayed Base Substitution Mutations During Error-prone or SOS Synthesis of Double-stranded DNA Containing Thymine and Adenine Molecules in T_3*, A_2* and A_4* Rare Tautomeric Forms

As I have shown [58–62], the source of the so-called untargeted mutations are DNA bases in rare tautomeric forms. The rare tautomeric forms of bases will be stable if these bases are located in small (3-5 bases) neighborhoods from DNA damage, for example, cyclobutane pyrimidine dimers and during DNA synthesis [61].

Bases bonded to each other by hydrogen bonds can change their tautomeric states if one or more hydrogen atoms pass in H-bonds [57]. If, the lengths of the hydrogen bonds increase, then a second minimum appears [102] and the hydrogen atoms cannot return to their previous positions. In other words, the bases will change their tautomeric states, they will turn into rare tautomeric states, and they are stable [57]. Of course, they will be stable in all cases when the DNA strand opposite the damage is bent. Consequently, only bases in rare tautomeric forms, when H-bonds between the bases are lengthened or even torn, can lead to untargeted mutations.

As I have shown [32,52,62-66], *cis-syn* cyclobutane pyrimidine dimers, one or both of which are in certain rare tautomeric forms, lead to targeted delayed base substitution mutations. Moreover, under certain conditions, even canonical *cis-syn* cyclobutane pyrimidine dimers can cause targeted delayed base substitution mutations [32,52,62,63,65,66]. It turned out that bases in tautomeric forms can lead to delayed mutations only if such bases can form both canonical base pairs and non-canonical base pairs [32,52,62-66].

Therefore, let us examine what mutations can appear opposite thymine and adenine molecules in the T_3*, A_2* and A_4* rare tautomeric forms. DNA bases in rare tautomeric forms can appear upon irradiation of a DNA molecule with ultraviolet light [103]. Mutations are always formed during DNA synthesis in error-prone or SOS replication, repair, or transcription processes [104-111].

Let's explore of the canonical bases incorporation opposite matrix bases, based on the fact [26] that specialized and modified DNA polymerases insert such canonical bases opposite matrix bases that are capable of forming H-bonds with matrix bases. As can be seen from Figure 1c, thymine T_3* can form one hydrogen bonds with adenine, guanine (Figure 1d), cytosine (Figure 1e) and thymine (Figure 1f). Adenine in the A_2* rare tautomeric form can form one hydrogen bond with thymine (Figure 2c), guanine (Figure 2e), cytosine (Figure 2d) and adenine (Figure 2f). Adenine in the A_4* rare tautomeric form can form one hydrogen bond with thymine (Figure 3c). But it can form one hydrogen bond with guanine (Figure 3d).

Consider a DNA site (Figure 4a), one strand of which contains one canonical *cis-syn* cyclobutane thymine dimer TT, and in a small vicinity of it there is thymine in the T_3* rare tautomeric form (Figure 1b), adenine molecules in A_2* (Figure 2b) and A_4* rare tautomeric forms (Figure 3b), as well as canonical thymine. Let other *cis-syn* cyclobutane pyrimidine dimers and other damage to the DNA molecule be quite far from it.

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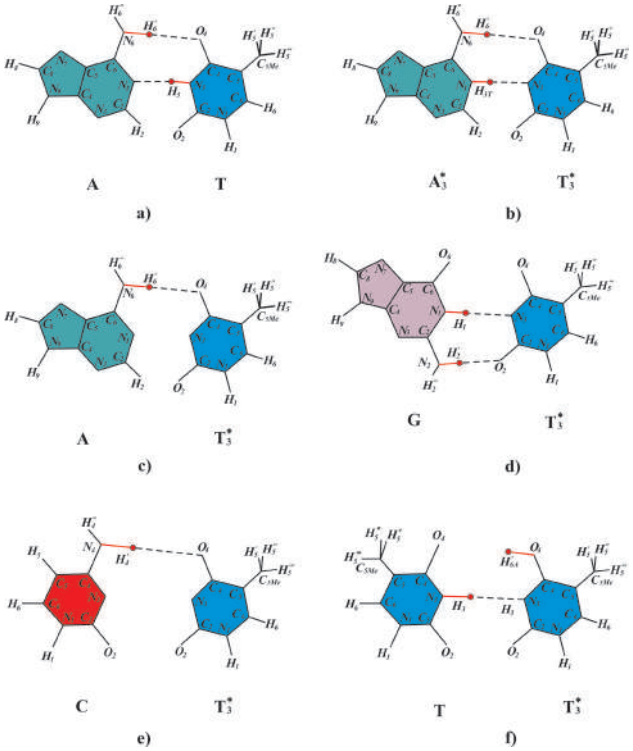

Figure 1. Rare tautomeric state of T_3* thymine and structural analysis of pairing of thymine T_3* with canonical DNA bases

Note: (a) thymine (T) and adenine (A) are in canonical tautomeric forms; (b) rare tautomeric forms of thymine T_3* and adenine A_3*; (c) - (f) structural analysis of pairing of thymine T_3* with canonical DNA bases: (c) with adenine; (d) with guanine; (e) with cytosine; (f) with thymine.

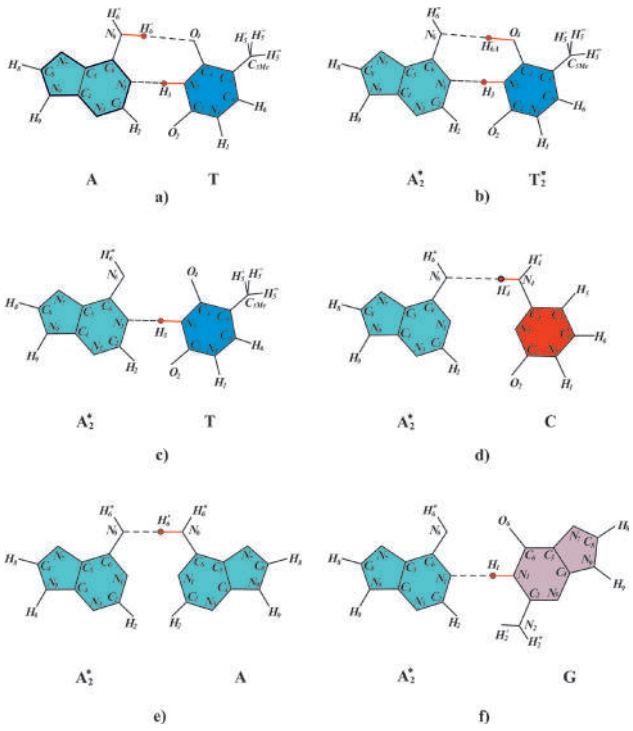

Figure 2. Rare tautomeric state A_2* of adenine and structural analysis of pairing of adenine A_2* with canonical DNA bases

Note: (a) thymine (T) and adenine (A) are in canonical tautomeric forms; (b) rare tautomeric forms of adenine A_2* and thymine T_2*; (c) - (f) structural analysis of pairing of adenine A_2* with canonical DNA bases: (c) with thymine; (d) with cytosine; (e) with adenine; (f) with guanine.

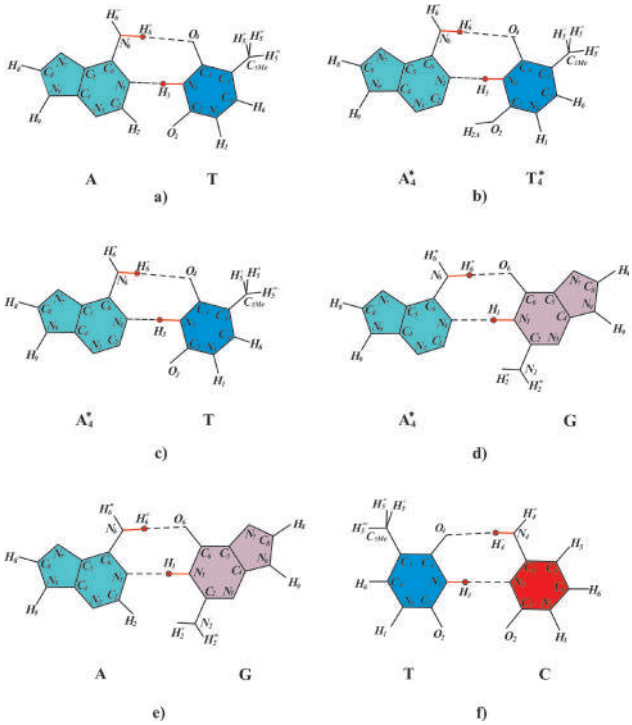

Figure 3. Rare tautomeric state A_4* of adenine and structural analysis of pairing of adenine A_4*, canonical adenine and thymine with canonical DNA bases

Note: (a) thymine (T) and adenine (A) are in canonical tautomeric forms; (b) rare tautomeric forms of adenine A_4* and thymine T_4*; (c) structural analysis of pairing of adenine A_4* with canonical thymine; (d) structural analysis of pairing of adenine A_4* with cytosine; (e) structural analysis of pairing of canonical adenine with canonical thymine; (f) structural analysis of pairing of canonical thymine with canonical cytosine.

Since damage capable of stopping DNA synthesis is only one, translesion synthesis will be carried out using DNA polymerase conduct error-free DNA synthesis. Adenine will be inserted opposite thymine T_3*. In this case, the mutation does not form (Figure 4c). For the same reasons, thymine will be inserted opposite adenine in rare tautomeric forms A_2* and A_4* (Figure 4b). In this case, mutations also do not form (Figure 4c). So many cycles of DNA replication can continue. Mutations will not appear until the situation changes.

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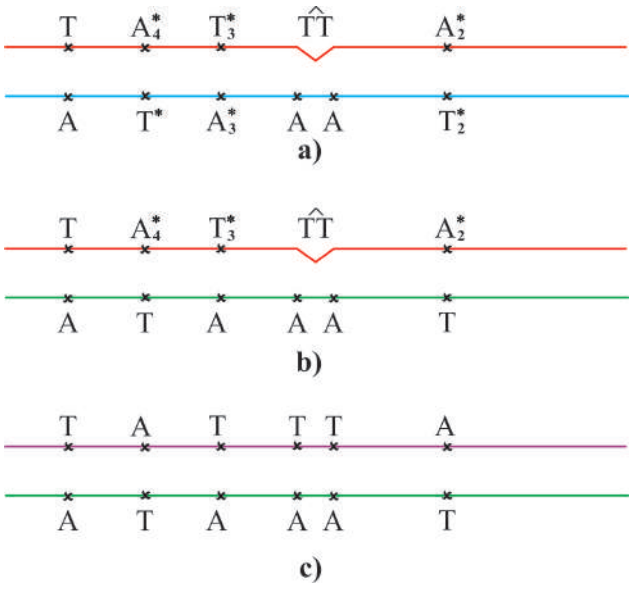

Figure 4. Error-prone or SOS-replication of the DNA containing canonical thymine, thymine in rare tautomeric form T_3*, molecules of adenine in rare tautomeric forms A_2* and A_4* located in a small neighborhood of the *cis-syn* cyclobutane thymine dimer TT. Molecules of thymine in rare tautomeric form T_3*, molecules of adenine in rare tautomeric forms A_2* and A_4* do not result in mutations

Note: (a) a DNA site containing canonical thymine, thymine in rare tautomeric form T_3*, molecules of adenine in rare tautomeric forms A_2* and A_4* located in a small neighborhood of the *cis-syn* cyclobutane thymine dimer TT; (b) adenine molecules are inserted opposite the thymine in the rare tautomeric form of T_3* and canonical thymine T, molecules of thymine are inserted opposite the adenine in rare tautomeric forms A_2* and A_4*; (c) molecules of thymine are inserted opposite molecules of adenine, molecules of adenine are inserted opposite molecules of thymine. Mutations do not form.

Suppose that after some, possibly, a long time, near the *cis-syn* cyclobutane dimer TT, another damage appears that can stop DNA synthesis. It can be caused, for example, by free radicals, the main cause of spontaneous mutagenesis. In Figure 5, it is indicated as Sp. In this case, the synthesis will continue to be carried out using error-free DNA and a sliding clamp. Let us assume that in this case the control over the formation of pyrimidine-purine bases pairs only will remain. Therefore, opposite thymine T_3*, with some probability, guanine can be incorporated (Figure 5b). This will lead to the formation of untargeted delayed transition T-A → C-G (Figure 5c). For the same reasons, cytosine will be inserted opposite adenine in the A_2* rare tautomeric form (Figure 5b). This will lead to the formation of untargeted delayed A-T→G-C transition (Figure 5c).

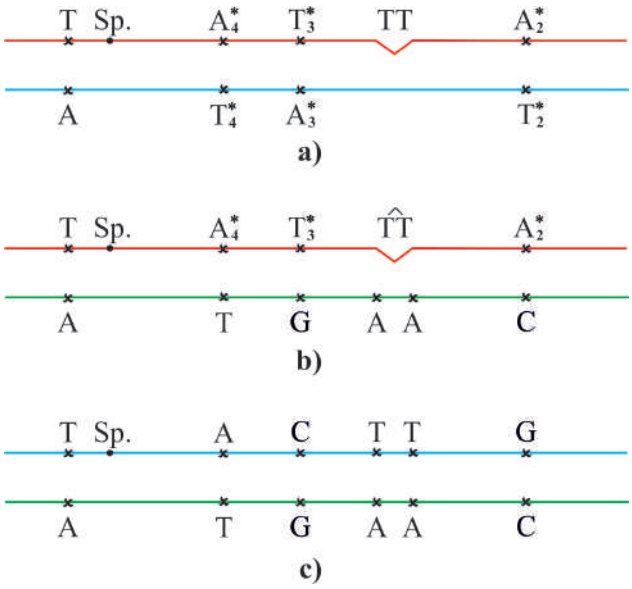

Figure 5. Error-prone or SOS-replication of the DNA containing canonical thymine, thymine in rare tautomeric form T_3*, molecules of adenine in rare tautomeric forms A_2* and A_4* located in a small neighborhood of the *cis-syn* cyclobutane thymine dimer TT and damage Sp. capable of stopping the synthesis of DNA. Thymine T_3* result in untargeted T-A → C-G transition, adenine A_2* result in untargeted A-T→G-C transition, adenine A_4* do not result in mutations

Note: (a) a DNA site containing canonical thymine, thymine in rare tautomeric form T_3*, molecules of adenine in rare tautomeric forms A_2* and A_4* located in a small neighborhood of the *cis-syn* cyclobutane thymine dimer TT and damage Sp. capable of stopping the synthesis of DNA; (b) a guanine is inserted opposite thymine T_3*, a cytosine is inserted opposite adenine A_2*, a thymine is inserted opposite adenine A_4*, molecules of adenine are inserted opposite molecules of canonical thymine T; (c) complementary base pairing occurs.

Let, after some time, many other damages appear near to the dimer TT. They can be caused by free radicals and some other chemicals (Ch.) [114]. Synthesis will be carried out using some specialized DNA polymerases and a sliding clamp. Then transitions and transversions can be formed.

Canonical cytosine can be incorporated opposite thymine T_3* (Figure 6b) and untargeted delayed T-A→G-C transversion form. The insertion of a canonical thymine opposite thymine T_3* (Figure 6b) produces untargeted delayed homologous T-A→A-T transversion.

For the same reasons, guanine can be inserted opposite adenine A_2* (Figure 6b). This will lead to the formation of a untargeted delayed A-T→C-G transversion (Figure 6c). In addition, opposite A_2*, adenine can be inserted (Figure 6b), which results in the formation of a untargeted delayed A-T→T-A transversion (Figure 6c). Guanine can be inserted opposite adenine A_4* (Figure 6b). This will lead to the formation of a untargeted delayed A-T→C-G

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transversion (Figure 6c).

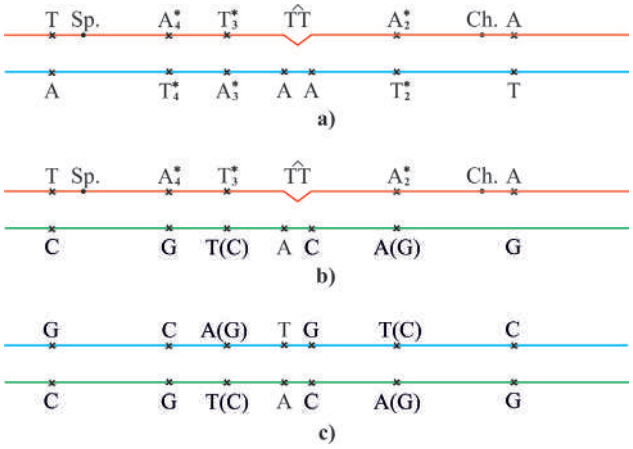

Figure 6. Error-prone or SOS-replication of the DNA containing canonical thymine, thymine in rare tautomeric form T_3*, molecules of adenine in rare tautomeric forms A_2* and A_4* located in a small neighborhood of the *cis-syn* cyclobutane thymine dimer TT and damages Ch. and Sp. capable of stopping the synthesis of DNA. Thymine T_3* result in untargeted transversion T-A→G-C or untargeted homologous transversion T-A→A-T, adenine A_2* result in untargeted transversion A-T→C-G or untargeted homologous transversion A-T→T-A, adenine A_4* result in untargeted transversion A-T→C-G, canonical thymine result in untargeted transversion T-A→G-C

Note: (a) a DNA site containing canonical thymine, thymine in rare tautomeric form T_3*, molecules of adenine in rare tautomeric forms A_2* and A_4* located in a small neighborhood of the *cis-syn* cyclobutane thymine dimer TT and damages Ch. and Sp. capable of stopping the synthesis of DNA; (b) a canonical cytosine or canonical thymine is inserted opposite thymine T_3*, a guanine or adenine is inserted opposite adenine A_4*, a cytosine is inserted opposite canonical thymine; (c) complementary base pairing occurs.

4. Polymerase-tautomeric Model for Untargeted Delayed Base Substitution Mutations during Error-prone or SOS Synthesis of Double-stranded DNA Containing Thymine and Adenine Molecules in Canonical Tautomeric Forms

Let's see if, under certain conditions, canonical thymine and adenine result in untargeted delayed mutations. This is a very important issue, since DNA molecules are usually made up of canonical bases, and damaged bases are quite rare. Of course, thymine can form a pair with adenine (Figure 3a). The thymine cannot form hydrogen bonds with guanine or thymine. But the thymine can form hydrogen bonds with the cytosine (Figure 3c). Of course, the adenine can form a pair with the thymine (Figure 3a). In addition, canonical adenine can form hydrogen bonds with canonical guanine (Figure 3d). These facts have long been known.

If there is only one cyclobutane pyrimidine dimer (Figure 4a) or one cyclobutane pyrimidine dimer and DNA damage caused by free radicals (Figure 5a), then adenine will be inserted opposite thymine T, and canonical thymine will be inserted opposite canonical adenine (Figure 4b, 5b). Mutations do not form (Figures 4c, 5c). And so many DNA replication cycles can go on.

Suppose that after some, possibly a long time, several other cyclobutane pyrimidine dimers were formed near the *cis-syn* cyclobutane dimer TT (Figure 5a). In this case specialized or modified DNA polymerase replicated past a *cis-syn* cyclobutane cytosine dimer with less accuracy. Let us assume that in this case the accuracy of control over the number of hydrogen bonds formed between the DNA bases will decrease. But control over the formation of pyrimidine-purine base pairs will continue. And in this case, adenine will be inserted opposite the canonical thymine (Figure 5b) and canonical thymine will be inserted opposite the canonical adenine and mutations will not form (Figure 5c).

Assume that after some time after DNA irradiation with ultraviolet light, near the canonical cis-sin cyclobutane thymine dimer TT, many other damages capable of stopping the DNA synthesis appear. Some of them can be caused, for example, by free radicals, the main cause of spontaneous mutagenesis. In Figure 6, I marked them as Sp. In addition, it may be other DNA damage that may be due to the action of some other chemicals that can damage the DNA. It can be heavy metals or other substances that can damage a DNA molecule. They were experimentally detected in patients with cardiovascular and cancer diseases [114]. In Figure 6, I marked them as Ch.

As shown by experiments, if there is a large amount of DNA damage, DNA polymerases with lower speed and accuracy are involved in the translesion synthesis. In the case DNA polymerases replicated past cyclobutane dimers and other damages are highly error-prone. Most likely, specialized DNA polymerase will be pressed by a sliding clamp. Only in this case transversions can form. Cytosine may be inserted opposite thymine T (Figure 6b). In this case, transversion A-T→C-G will appear (Figure 6c). Canonical guanine may be inserted opposite canonical adenine. In this case, transversion A-T→C-G will appear (Figure 6c).

5. The Nature of Untargeted Delayed base Substitution Mutations

It can be concluded that thymine molecules in the rare

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tautomeric form T_3*, which can form hydrogen bonds with both adenine and other canonical DNA bases, can be the source of untargeted delayed base substitution mutations. Adenine molecules A_2* and A_4*, which can form hydrogen bonds with thymine and other canonical DNA bases, can also be the source of untargeted delayed base substitution mutations. Canonical thymine and adenine can also lead to untargeted delayed base substitution mutations. Whether or not an untargeted delayed base substitution mutation appears, is completely dependent on the neighboring environment.

If next to the thymine T_3* or the adenine in the rare tautomeric form A_2* or A_4* there are no other DNA damages or there are very few of them, then synthesis through the damage will proceed quite accurately and no mutations will form. If next to the thymine in the rare tautomeric form T_3* or the adenine in the rare tautomeric form A_2* or A_4* there are other lesions that can stop DNA synthesis, then the synthesis will be carried out using specialized DNA polymerases with low accuracy of synthesis. DNA synthesis can also occur with the help of constitutive DNA polymerases, but provided that they are pressed with a sliding clip. As a result, the thymine T_3*, can cause untargeted delayed T-A→C-G transition.

And, if near the thymine T_3* or the adenine in the rare tautomeric form A_2* or A_4* there will be many damages that can stop DNA synthesis, specialized DNA polymerases with very low accuracy will be involved in the translesion synthesis. In addition, their accuracy can be reduced by the operation of a sliding clamp. Thymine T_3* can lead to a untargeted delayed T-A→G-C transversion or a untargeted delayed homologous T-A→A-T transversion. The adenine A_2* can lead to the untargeted delayed A-T→C-G transversion and untargeted delayed homologous A-T→T-A transversion. The adenine A_4* can lead to the formation of a untargeted delayed A-T→C-G transversion.

If there is a lot of damage on the DNA site that can stop DNA synthesis, then the thymine molecule in the canonical tautomeric form can lead to the T-A→G-C transversion only, and the adenine canonical tautomeric molecule can lead to the A-T→ C-G transversion only.

6. Contribution of untargeted delayed base substitution mutations to cancer risk

Typically, mutations that lead to cancer are divided into mutations caused by hereditary factors and caused by environmental factors. Tomasetti and Vogelstein [112] suggested that there is a third source of mutations, these mutations appear as a result of random errors that occur during normal DNA replication. In Ref. [112], it was concluded that only a third of cancer risk among tissues is associated with environmental factors or inherited predispositions. But basically, the risk of malignant tumors is due to random mutations that occur during normal DNA replication. In other words, according to the cancer risk model [112], the formation of about 67% of all mutations is not caused by exposure to any mutagens. The authors conclude that no cancer prevention measures can affect this part of mutagenesis [112].

In the currently accepted polymerase paradigm of mutagenesis, it is believed that targeted mutations appear opposite to damage that can stop DNA synthesis [6,7,21–25]. It is believed that untargeted mutations form on non-damaged DNA sites [6,8-13]. The nature of untargeted mutations is not understood [3,20,43,44]. The nature of delayed mutations is not known [3,45]. Therefore, according to the polymerase paradigm, only some of all mutations can form opposite to lesions that can stop DNA synthesis. Therefore, the conclusions of Tomasetti and Vogelstein [112], in principle, do not contradict the generally accepted polymerase paradigm of mutagenesis. In order to test the hypothesis of Tomasetti and Vogelstein [112], we compare the conclusions drawn in this cancer risk model with some experimental data on studies of untargeted delayed mutations.

More than half of delayed mutations are base substitution mutations [88]. Experiments show that when combined with 8-methoxy-psoralen and long-wave ultraviolet light, about 90% of the induced mutations were untargeted delayed mutations [113]. As shown in this paper, untargeted delayed mutations appear opposite DNA bases in certain rare tautomeric forms. These rare tautomeric forms of DNA bases can appear only under the influence of some external factors, for example, exposure to a DNA molecule with ultraviolet light or some chemicals. Moreover, these rare tautomeric forms will be stable only under certain conditions. They will be preserved only when the DNA strand opposite the corresponding bases is bent so that the hydrogen bonds between the bases are lengthened or torn. Then the hydrogen atoms will not be able to return to their previous positions. A number of studies have shown that the DNA strand bends opposite cyclobutane pyrimidine dimers [78–82].

Therefore, in order for untargeted delayed mutations to form, several independent DNA lesions are necessary. Firstly, the action of a substance is necessary, which will lead to strong forced vibrations of the bases bonded by hydrogen bonds, which can lead to a change in the position

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of one or more hydrogen atoms [57]. Secondly, an action is needed that will lead to another DNA damage that will cause the DNA strand to bend [26]. But this is not enough. Thirdly, it is necessary that other DNA damages appear nearby, which will lead to the induction of an error-prone or SOS system [65]. In other words, it is necessary that DNA synthesis proceeds using specialized DNA polymerases, characterized by low accuracy of synthesis. Such damage can be formed under the influence of free radicals that appear in the processes of metabolism or other chemicals. These can be heavy metals or other substances that have been found in patients with cardiovascular and cancer diseases [114].

We see that, at least with regard to untargeted delayed mutations when they are formed when combined with 8-methoxy-psoralen and long-wave ultraviolet light, the hypothesis of Tomasetti and Vogelstein [112] that about 67% of all mutations are formed not caused by exposure to any mutagens, does not withstand any criticism. As the experiment shows, in this case under combined do with 8-methoxy-psoralen and long-wave ultraviolet light about 90% of the induced mutations were untargeted delayed mutations [113]. And as the polymerase-tautomeric model shows, in order to form such mutations, the formation of several independent DNA lesions is necessary. Moreover, part of these lesions should lead to very significant effects, namely, will cause the DNA strand to bend and the induction of specialized DNA polymerases. In experiment [113], long-wave ultraviolet light can lead to a change in the tautomeric states of DNA bases, and 8-methoxy-psoralen molecules can lead to bending of the DNA strand and induce an error-prone or SOS system.

As shown in this paper, under certain conditions, even canonical thymine or adenine can lead to mutations. This is possible when many different DNA lesions are formed, which causes not only the induction of specialized DNA polymerases, but also the work of a sliding clamp, it presses specialized DNA polymerases to template DNA, resulting in a large number of mutations.

For the untargeted delayed mutations formation, the appearance of several DNA damage is necessary. In fact, for the untargeted delayed mutations formation, significantly more DNA damage is required than with the formation of targeted mutations. Therefore, the assumption made in the cancer risk model [112] that the formation of about 67% of all mutations is not caused by exposure to any mutagens is erroneous, at least with respect to untargeted delayed base substitution mutations. In addition, the cancer risk model [112] contradicts the experimental data obtained in Ref. [113].

The authors of the cancer risk model [112] conclude that no cancer prevention measures can affect this part of mutagenesis. This conclusion, in my opinion, is also not true. I believe that for cancer patients it's not at all hopeless, as the authors of the work [112] try to assure us. The strategy is pretty obvious. It is necessary to find out in what form heavy metals and other substances that we received with air, water and food are. A method must be developed for their removal and removal. As soon as we reduce the mutagenic and damaging load on DNA molecules, it is quite possible the body will cope with the tumor. Maybe, you may need help to ensure that all body systems work.

7. Conclusion

At present, mechanism of delayed mutations formation is not clear [3]. In polymerase model it is assumed that sometimes DNA polymerases are inserted opposite the matrix bases, for example, those included in the composition of cyclobutane pyrimidine dimers, such canonical bases that cannot form hydrogen bonds with the matrix bases [24]. I have proposed and are developing models for targeted [26,31,33,38-42,52-57,115], untargeted [58-62], and delayed mutagenesis [32,52,62-66]. In this paper, I propose a mechanism for untargeted delayed base substitution mutations formation caused by thymine and adenine molecules. Untargeted delayed mutations are mutations that can appear after several cycles of replication after exposure to the mutagen on the so-called not damaged DNA sites. Thymine and adenine can form five rare tautomeric forms that are stable if the corresponding nucleotides are part of cyclobutane dimers or are located in small neighborhoods from them.

Error-prone and SOS synthesis of a DNA site, one strand of which contains one canonical *cis-syn* cyclobutane thymine dimer TT, and in a small vicinity of it there is thymine in the T_3* rare tautomeric form, adenine molecules in A_2* and A_4* rare tautomeric forms, as well as canonical thymine and canonical adenine. Opposite thymine T_3*, adenine can be incorporated, but may be inserted any other canonical base. Opposite adenine in rare tautomeric form of A_2*, thymine can be incorporated, but guanine or adenine may be inserted. Opposite adenine A_4* thymine can be incorporated, but guanine may be inserted.

If next to thymine T_3*, adenine A_2* or A_4* there are no other DNA damages or there are a few of them, then synthesis through the damage will proceed quite accurately and mutations will not form.

If in the small neighborhood of the thymine in the rare tautomeric form T_3* or the adenine in the rare tautomeric

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form A_2* or A_4* there are other damages that can stop DNA synthesis, then the synthesis will be carried out using specialized DNA polymerases with low synthesis accuracy. DNA synthesis can also occur with the help of constitutive DNA polymerases, but provided that they are pressed with a sliding clamp. As a result, the thymine in the rare tautomeric form T_3* can cause a untargeted delayed T-A→C-G transition, and the adenine molecules A_2* or A_4* will not lead to a mutation.

If in the small neighborhood of the thymine in the rare tautomeric form T_3* or the adenine in the rare tautomeric form A_2* or A_4*, specialized DNA polymerases with very low accuracy of synthesis will be involved in the synthesis through damage. Moreover, their accuracy may be reduced by the operation of a sliding clamp. In this case, the thymine in the rare tautomeric form T_3* can cause T-A→C-G untargeted delayed transition, and can lead to T-A→G-C untargeted delayed transversion or T-A→A-T untargeted delayed homologous transversion. The adenine in the rare tautomeric form of A_2* can lead to the formation of untargeted delayed A-T→C-G transversion and untargeted delayed A-T→T-A homologous transversion. The adenine A_4* can lead to the formation of a untargeted delayed A-T→C-G transversion.

The thymine in canonical tautomeric form can lead to untargeted delayed T-A→G-C transversion only, and the adenine in canonical tautomeric form can lead to untargeted delayed A-T→ C-G transversion only.

It is concluded that thymine in the T_3* rare tautomeric form, which can form hydrogen bonds with both adenine and other canonical DNA bases, can be the source of untargeted delayed base substitution mutations. In addition, adenine molecules in the rare tautomeric forms A_2* and A_4*, which can form hydrogen bonds with thymine and other canonical DNA bases, can also be a source of untargeted delayed base substitution mutations. In addition, thymine and adenine in canonical tautomeric forms can also lead to untargeted delayed base substitution mutations. Whether or not untargeted delayed base substitution mutation appears, is completely dependent on the neighboring environment. Not all of these damage must be mutagenic. If these lesions are able to stop DNA synthesis, then, therefore, they can lead to synthesis through damage, cause DNA polymerase with low synthesis accuracy and, therefore, contribute to mutagenesis.

As shown earlier, the formation of five rare tautomeric forms of thymines or adenines is possible. If they are located in a small vicinity of the cyclobutane pyrimidine dimer or other damage causing the DNA strand to bend, then these rare tautomeric states will be stable. Each of these bases in rare tautomeric forms can lead to certain types of untargeted mutations. Thus, thymine T_1*, T_4* and T_5*and adenine in the rare tautomeric form A_1* can cause untargeted base substitution mutations [32,52,60,61] only. Thymine T_2* can lead to untargeted frameshift mutations only, for example, to untargeted insertions [32,62]. Thymine in the T_3* rare tautomeric form can cause untargeted delayed base substitution mutations only. The thymine in the T_3* rare tautomeric form can cause T-A→C-G untargeted delayed transition, T-A→G-C untargeted delayed transversion or T-A→A-T untargeted delayed homologous transversion. The adenine in the A_2* rare tautomeric form can lead to the formation of untargeted delayed A-T→C-G transversion and untargeted delayed A-T→T-A homologous transversion. The adenine in the A_4* rare tautomeric form can lead to the untargeted delayed A-T→C-G transversion. The canonical thymine can lead to untargeted delayed T-A→G-C transversion only, and the adenine in canonical tautomeric form can lead to untargeted delayed A-T→ C-G transversion only.

I developed models for targeted base substitution mutations [26,32,33,52,55,66], targeted insertions [32,33,39,66], targeted deletions [32,33,40,41,66], targeted complex insertions [32,33,42,66], delayed targeted base substitution mutations [63,64-66]. I developed models for untargeted mutations [32, 58–62,66] such as untargeted insertions [32,62], untargeted base substitution mutations [32,58–61,66] that appear immediately after irradiation, and untargeted delayed base substitution mutations. The polymerase-tautomeric models of radiation-induced genomic instability [32,52,63-66] are able to explain such phenomena of radiation-induced genome instability as targeted delayed insertions [32,62,66], targeted delayed base substitution mutations [32,52,63,64,66] and untargeted delayed base substitution mutations.

Experimental studies [27,28] provides strong support for the ideas of Watson and Crick [30] and the polymerase-tautomeric models for mutagenesis through direct structural evidence. Thus it is need to change the paradigm in mutagenesis.

The source of untargeted delayed base substitution mutations is thymine in the T_3* rare tautomeric form and adenine in the A_2* and A_4* rare tautomeric forms. But even if such DNA damage appears, in most cases they will not lead to the appearance of mutations. In order for untargeted delayed mutations to form, it is necessary that there be other DNA damage. Opposite some lesions, the DNA strand must be bent, while other lesions should be able to stop DNA synthesis.

Since, under the combined action of 8-methoxy-psoralen and long-wave ultraviolet light, about 90% of the induced mutations were untargeted delayed mutations [113], in this case, with the onset of cancer, at least 90% of the mutations

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were formed as a result of DNA damage. Long-wave ultraviolet caused the appearance of bases in rare tautomeric forms, and 8-methoxy-psoralen led to a curvature of the DNA strand and, as a result, stabilization of these rare tautomeric forms of DNA bases. In addition, 8-methoxy-psoralen led to induction of error-prone or SOS system.

Therefore, the conclusion drawn from the cancer risk model [112] that the formation of about 67% of all mutations is not caused by exposure to any mutagens, but is the result of normal replication, is erroneous. As we can see from the example of the untargeted delayed mutations formation, all these mutations can appear during the induction of error prone or SOS systems only. Moreover, the synthesis should occur using specialized DNA polymerases, and even the work of a sliding clamp. This is only possible when the synthesis of DNA containing a lot of damage occurs. Therefore, the conclusion of the cancer risk model [112] that the formation of 67% of all mutations is not caused by exposure to any mutagens, but occurs during normal DNA replication, is erroneous. It contradicts experimental facts. Naturally, the conclusion of the cancer risk model [112] that no cancer prevention methods can prevent 67% of all mutations is certainly wrong.

The authors of the cancer risk model [112] conclude that no cancer prevention measures can affect this part of mutagenesis. This conclusion, in my opinion, is also not true. I believe that for cancer patients it's not at all hopeless, as the authors of the work [112] try to assure us. The strategy is pretty obvious. It is necessary to find out in what form heavy metals and other substances that we received with air, water and food are. A method must be developed for their removal and removal. As soon as we reduce the mutagenic and damaging load on DNA molecules, it is quite possible the body will cope with the tumor. Maybe, you may need help to ensure that all body systems work. I hope that a deeper understanding of the mechanisms of mutations formation, and, consequently, a deeper understanding of the mechanisms of cancer formation, will allow us to develop more effective methods for the prevention and treatment of cancer.

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