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Research progress on the application of CRISPR/Cas9 technology in breast cancer treatment

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Abstract. Cancer is viewed as a severe health issue because it is one of the leading causes of mortality on a global scale. The most frequent type of cancer in women and one of the leading causes of mortality worldwide is breast cancer. Current treatments include surgical excision, hormone treatment, chemotherapy, radiotherapy, and targeted biological therapy. Although numerous treatments can relieve the symptoms or even cure the disease. Several genes are still waiting for discovery that affects the growing processes of tumors through a different pathway. Continuous effort is needed to develop more efficient and fewer side effect treatments. The genetic intervention has arisen as one of the most auspicious therapeutic alternatives in contemporary times. "CRISPR Cas9 has emerged as a compelling gene editing technology in the contemporary era, which is constructed by two main parts-the CRISPR-associated (Cas) endonuclease section and the CRISPR RNA (crRNA) section. CRISPR technology has been successfully utilized to establish gene-specific cell lines and animal models for studying gene function and their products in pathogenesis, as well as interactions between different genes. This paper summarizes the recent years of experiments using CRISPR/Cas9 technology to explore the mechanism of breast carcinogenesis, find appropriate therapeutic targets, and discuss the latent capacity of CRISPR/Cas9 technology in breast cancer treatment.

Keywords: breast cancer, CRISPR-Cas9, triple-negative breast cancer.

1. Introduction

Breast cancer ranks among the leading causes of mortality across the globe, with a tendency to metastasize to organs such as bone, liver, and lungs. Additionally, it is the most common form of cancer among women [1]. The fifth most common cancer in the world is breast cancer, which is nearly 100 times more common in women than previously recognized. There were 2.3 million new instances of breast cancer in 2020, representing 11.7% of all newly diagnosed cancers. with a high incidence of 226,419 cases and 678,996 deaths, ranking first in most countries in terms of incidence and mortality [2]. Among Chinese women, breast cancer accounts for 16.72% of all new cancer cases (306,000) [3].

Clinically, there are four categories of breast cancer: triple-negative, HER2-positive, luminal A, and luminal B. (TNBC). The five main breast cancer risk factors include aging, familial inheritance (e.g., mutations in genes such as BRCA1, BRCA2, c-erbB-2, EGFR, c-Myc), reproductive factors (e.g., irregular menstrual cycles, delayed first pregnancy age, and poor fecundity), estrogen (ovarian secretion,

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oral contraceptives, HRT, and other contraceptive methods) and lifestyle (e.g. alcohol abuse, excessive dietary fat intake), etc [1].

Mammography and MRI are presently employed to screen for breast cancer, while chemoprevention uses estrogen receptor modulators (SERMS) and aromatase inhibitors (AI), of which triamcinolone acetonide (TAM) is one of the best-known SERMS drugs and has been used in clinical treatment for more than 30 years. Bio prevention, on the other hand, mainly uses monoclonal antibodies with HER2 as one of the main targets, such as trastuzumab (Herceptin) and pertuzumab (Perjeta), as well as the PD1 inhibitor drugs Nivolumab (Opdivo) and Pembrolizumab (Keytruda) [1]. However, the benefit of certain drugs is limited to a small number of patients. For example, the objective response rate of the PD-1 checkpoint inhibitor monoclonal antibody Pembrolizumab in advanced TNBC expressing PD-L1 was only 18%. Therefore, finding novel immunological targets for the therapy of breast cancer is crucial [4].

Breast cancer usually develops due to abnormalities that have accumulated in local epithelial cells, genetic mutations, and dysregulation of signaling pathways, leading to complex genomic and heterogeneous tumor formation [4,5]. However, breast cancer's etiology is very complex, and its specific pathogenesis has not been fully elucidated [1]. Therefore, the treatment of breast cancer remains a challenge. A thorough understanding of the involvement of these pathogenic genes in breast cancer carcinogenesis is required to identify improved therapeutic approaches and targets. Therefore, the establishment of genetic screening networks related to breast cancer using advanced genetic techniques is essential to meet the progressively growing need for alternative treatments and more effective therapeutic approaches [4].

2. CRISPR/Cas9 system

The biological and therapeutic disciplines have shown great promise for clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-associated protein (CRISPR/Cas9) technologies in previous years. The method has been effectively used to create animal models and cell lines specific for studying the function of various genes and the interactions between genes and their products in disease. Aside from that, CRISPR/Cas9 technology can be utilized to identify novel immuno-oncology targets, develop novel tumor immunomodulators, and analyze their mechanisms [4].

CRISPR-Cas technology originated from the bacterial immune system. While researching Streptococcus thermophilus in 2005, Bolotin et al found an atypical CRISPR locus and triumphantly identified novel Cas9 genes. They also mentioned that the CRISPR-Cas9 system target identification must be accompanied by the protospacer region ringing gene sequence (PAM) [6]. After continuous development, CRISPR/Cas9 emerged as a revolutionary and powerful instrument that helps to correct, insert or delete genetic material in both laboratory and animal models [7].

The CRISPR-associated (Cas) endonuclease section and the CRISPR RNA (crRNA) section are two modules that make up the CRISPR complex. This facilitates the modification of the separately encoded target section (sgRNA) and the nucleic acid endonuclease section (Cas9), as they can be optimized segregatively without altering each other's functions. Double-stranded DNA breaks are started by the CAS nucleic acid endonuclease module, which also specifies the crRNA module for the target DNA sequence. Uridine residues in the mitochondria of kinetic top-dividing protozoa mRNA can be added or removed under the control of gRNA [8]. Methods such as CRISPR knockout and CRISPRi based on CRISPR-Cas9 have been used to uncover significant oncogenes by screening very large libraries of sgRNA libraries [7].

The development of CRISPR-Cas9 technology has produced new opportunities for studying functional genomics. Genetic studies on faulty genes and their functions are now available because of technology that makes precise genome editing possible. In addition, It can be used to determine the genes that promote the activity of cancer cells and control cancer medication sensitivity [9]. The globe over, CRISPR-Cas9 technology has been effectively applied to target crucial genes in diverse model organisms and cell types, spanning from bacteria and mice to human cells [7]. Therefore, the application of CRISPR/Cas9 tools in vivo models can as a first step toward translational applications fix particular mutations that lead to cancer formation and progression.

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3. Application of CRISPR/Cas9 system in breast cancer

3.1. In vivo studies

Although the molecular mechanisms of some tumor microenvironments (TMEs) have not been elucidated, some cells in TMEs have been identified to encourage tumor development and metastasis. Experimental evidence has shown that tumor-associated macrophages (TAMs) are major players and are associated with tumor development [10]. This has motivated researchers to look for fresh targets for TME editing in tumor therapy., such as Cop1 and Lgals2.

Cop1 has been discovered to be crucial for tumorigenesis and devolution over the past ten years. Wang. X et al. constructed a mouse CRISPR knockout library (MusCK) using CRISPR-Cas9 technology and performed an in vivo CRISPR screen in 4T1 cells from homozygous BALB/c mice to confirm the dependability of this library's in vivo screening. The in vivo screen showed that Cop1 was the most significant missing gene in TNBC, and the gene Cop1 KO by CRISPR KO (Cop1 KO) TNBC cells showed significantly slower tumor progression in vivo and prolonged survival in mice. The experiments about Cop1 KO illustrate that Cop1 may be a target for immunotherapy [4].

Galactose lectin-2, a glycan-binding protein that is involved in myocardial infarction, collateral arteriogenesis, cell adhesion, and T-cell apoptosis, is encoded by the gene Lgals2. Ji P et al. knocked down the Lgals2 gene (Lgals2 KO) in cells using the CRISPR-Cas9 editing method, which was then implanted in vivo. The results revealed that the tumor volume gradually decreased until it nearly vanished at the end. The reduced trend of infiltrating cytotoxic T lymphocytes (CTL), cytotoxic factors, the NK cells' rate of proliferation and myeloid cells shown by cell flow analysis; the results of the decreased killing of CTL and NK cells; and the evidence suggests that Lgals2 mediates immunosuppression in TNBC through the tumor microenvironment in vivo and that Lgals2 KO has tumor suppressive effects in vivo. Lgals2 encourages M2-like divisiveness and multiplication of macrophages through stimulation of colony-stimulating factor 1 (CSF1)/CSF1 receptor (CSF1R) axis. Consequently, Lgals2 has the potential to be a viable drug target for triple-negative breast cancer (TNBC) [11].

One of the most frequent cancer-promoting pathways in human tumors is PI3 kinase/mTOR/AKT pathway mutational activation. Phosphatidylinositol 3-kinase (PI3K)/AKT and PI3K-mTOR signaling pathways modulate diverse cellular procedures that are involved in the tumor cell cycle, apoptosis, autophagy, and other biological processes, which in turn regulate the survival, growth, and proliferation of tumor cells. One of the most frequent routes that promote malignancy in human tumors is the PI3 kinase/AKT pathway [12].

CRISPR-Cas9 was used as an efficient new technology to generate isogenic AKT1 mutant breast models to reveal the pathway of AKT1 inhibition of tumorigenesis with AKT1 promoting tumorigenesis. In vivo experiments with CRISPR-Cas9 lentiviral CDH1 infection of p53ko/E17K breast carcinoma cells by Gao SP et al. demonstrated that CDH1 gene transcription is AKT1-dependent and that co-mutation of CDH1 with AKT1 E17K may abrogate cell metastasis caused by AKT1 E17K mutation. The correction of AKT1 E17K mutations offers a novel site for the therapy [12].

The gene TET1 maintains the PI3K/mTOR pathway's activity. The experiments that used the CRISPR technology to delete the TET1 gene are evidence for this conclusion. The TET1 may also be a target therapeutic site for TNBC due to the PI3K/mTOR pathway's vital position in the formation of TNBC [13].

Breast cancer development also involves the expression of the PERP protein. The Cullin-RING ligase 4 (CRL4) E3 ubiquitin ligase, which promotes tumorigenesis through a variety of mechanisms, has recently been identified substrate receptor for the DDB1-and CUL4-associated factor 13 (DCAF13), and DCAF13 is a novel RNA binding protein (RBP) that aids TNBC metastasis. The in vivo experiments of Huang H's group investigated the way DCAF13 regulates tumor progression in vivo by transplanting DCAF13 KO breast cancer cells into mice with the help of CRISPR KO technology. After excluding the upregulated gene SLC22A18 by the DCAF13 gene, Employing CRISPR/Cas9 technology, PERP-KO breast cancer cells were created, saving the cell proliferation and colony formation caused by DCAF13

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KO to some extent. With regards to apoptosis, cell-cell adhesion, the transition between the epithelium and the mesenchyme, and Ca2 + signaling, PERP plays several different roles in cancer. These findings imply that DCAF13 may stimulate breast cancer cell growth by repressing the expression of PERP [13]. It was demonstrated that DCAF13 proliferates in the vicinity of breast carcinoma cells by inhibiting the PERP activity fraction. This opens the possibility of developing drugs targeting the DCAF13 gene to treat cancer [14].

Exploring the regulatory pathways of T lymphocytes is essential for the therapy of breast cancer since the state of T cells is directly associated with the emergence of cancer. The T cell receptor (TCR) recognizes antigens presented by major histocompatibility complex (MHC) class I (MHC-I) molecules, activating pathogen-specific CD8 T cells and triggering their activation, leading to proliferation, cytokine secretion, and cytotoxicity against target cells. In contrast, immunopathology and autoimmune disease can result from dysregulated CD8+ T cell activation. According to clinical findings, DHX37 is linked to T-cell malfunction in patients suffering from breast carcinomatosis [15].

Dong MB et al. screened DHX37, a gene regulating CD8 T cell degranulation, in mice with the aid of a high-throughput CRISPR screen. sgRNA targeting DHX37 and other genes. was cloned into a T cell chronic viral CRISPR vector, cultured, engineered T cells, and transplanted into mice carrying E0771-mCh-OVA tumors. The results showed that in the Dhx37 gene-edited T-cell treatment group, tumors were strongly suppressed starting on day seven and the degree of tumor suppression was higher than in the other groups, proving that Dhx37 targeted by CRISPR improved the anti-cancer effects of OT-I. The group also used AAV-CRISPR T cell knockout vectors to infect metazoan mouse CD8 T cells for gene editing. Immunological characterization and co-culture experiments showed increased expression of various pathways that activate T cells in CD8 T cells that have the Dhx37 mutation; T cell activation-related genes were prominent in the increased gene set; and Dhx37 mutant ((AAV-sgDhx27-treated) CD8 T cells demonstrated superior efficacy in eliminating homologous cancer cells. The above results reveal that Dhx37 is likely to suppress cancer cells through T-cell activation. Thus, CRISPR technology implies that the immune activity of T cells can be enhanced by developing targeted drugs against the DHX37 gene [15].

In addition, the exploratory capabilities of CRISPR technology can be useful in a variety of ways, including finding suitable targets to enhance the efficacy of drugs. Anti-HER2 medications have been researched, and HER2 gene amplification and overexpression are observed in a significant proportion of breast cancer cases, ranging from 14% to 30%, which are known as HER2-positive breast cancer. But in individuals with HER2-positive cancer, anti-HER2 resistance has grown to be a significant factor in therapy failure. Previous studies have demonstrated anti-HER2 resistance due to FGFR2 and FGFR3, but research on FGFR4's part in anti-HER2 resistance is scarce, and the recurrence rate is higher in those with malignancies that express FGFR4 strongly; the FGFR4 expression is elevated in metastatic breast cancer compared to the primary tumors. Therefore, Zou Y et al. explored the role of FGFR4 in the therapy of HER2-positive breast cancer by silencing it with CRISPR. The genome-wide CRISPR screen in mice identified FGFR4 as a key factor in cancer anti-HER2 because of its highly functional and pathway enrichment and the good clinical results of drugs targeting FGFR4 in other tumors [16].

3.2. In vitro studies

The establishment of an overexpression model of a specific gene for in vitro proliferation by CRISPR can be further used to determine the pathway of influence of a certain gene on tumor proliferation, such as Lgals2, DCAF13, ASPH, and AKT1. In the study by Ji P et al, Lgals2 overexpression models for in vitro proliferation and in vivo proliferation, Lgals2 KO cells multiplied in vitro as opposed to in vivo. The discrepancy in the experimental results further confirmed that Lgals2-mediated immunosuppression may be dependent on TME [10]. Huang H et al. eliminated the DCAF13 gene in breast cancer cell lines (4T1 et al.) with the help of CRISPR and DCAF13 KO was discovered that lead to a reduction in cellular development and colony number formation, prevented breast cancer cell lines from migrating, triggered G1/S phase cell cycle arrest, thereby preventing the spread of breast cancer cells. This implies the potential of DCAF13 in antitumor therapy [13].

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Normal adult tissues lack Aspartate β-hydroxylase (ASPH), but 90.1 percent of breast carcinomatosis patients have detectable ASPH because It contributes significantly to generating and keeping the malignant phenotype. Exosomes enable the delivery and transport of the proto-oncogenic secretome for long-distance cellular communication. Utilizing the CRISPR-Cas9 to knock down ASPH, Lin Q et al. found that ASPH KO significantly reduced the invasive phenotype of MDA-MD-231 cells (certain expression of endogenous ASPH), indicating that ASPH is Pivotal for the invasion and dissemination of breast carcinoma cells. The results demonstrate that ASPH initiates metastasis of motile breast carcinoma cells through the Notch axis in ASPH effector MMPs/ADAMs mediating ECM degradation or remodeling and that SMI that specifically counteracts the β-hydroxylase activity of ASPH reverses these aggressive phenotypes, suggesting that ASPH is a possible remedial target for breast cancer's complex metastases of breast carcinoma [17].

Other in vitro experiments targeting the gene include Gao SP et al., who targeted the AKT1 gene's E17K mutation. Knock outing AKT1 E17K after silencing TP53 by CRISPR KO produced p53ko/AKT1 E17K MCF-10A cells (p53ko/E17K). p53ko/E17K cells rescued the reduced proliferation of p53ko cells and even restored it to a level higher than that of the parental cells so that Human mammary epithelial cells might acquire an untransformed oncogenic state if AKT1 E17K is expressed. Compared to 53ko cells, significantly less migratory and invasive capability existed in p53ko/E17K cells, and the restoration and downregulation of EMT markers (E calreticulin and ZEB1) were associated, whereas epithelial-mesenchymal transition (EMT) is usually closely linked to, among others, the migration and invasiveness of tumor cells. Thus activated AKT1 inhibits cell migration by reversing epithelial-mesenchymal transition (EMT), based on a mechanism in which chelation of β-catenin with the cytomembrane leads to a downregulation in the transcription of ZEB1 and a concomitant upregulation in the production of E-calmodulin [14]. Those consequences provide a compelling justification for the creation of isozyme-selective AKT inhibitors as cancer treatments.

Although many studies have targeted specific genes and explored the mechanisms by which they affect tumorigenesis, Numerous genetic changes are what cause most malignancies., but the synergistic role of these changes in tumorigenesis is still unknown. To gain insight into the interactions between tumor suppressor genes (TSGs) regulating tumorigenesis. Zhao X et al. conducted a series of in vitro experiments using CRISPR KO technology to analyze how the combination of inactivated TSGs alters the tumorigenic growth characteristics as well as the transcriptome of mammary epithelial cells. single-cell transcriptome analysis of CRISPR double knockouts showed that the inactivation of cooperant TSGs leads to transcriptional up-regulation, which is not the case for non-cooperating TSGs. Such transcriptional alterations impact the expression of mediators involved in cancer formation and targeted treatment, such as CDK4, SRPK1, etc. TSGs like PTEN and TP53 are dually inactivated, for example, leading to altered epistatic expression. Gene enrichment analyses and prognostic analyses have also shown that a key factor driving the development of polygenic cancer is transcriptional epistasis, and that promoter binding to important transcription factors, which controls driver gene function, can also be related to transcriptional epistasis [18].

Dekkers JF et al. used CRISPR-Cas9 mediated gene editing of human breast tissue to mimic tumor formation. In the experiment, the researchers first introduced Cas9 into normal cells and then sequentially introduced unidirectional guide RNAs expressing the targeted PTEN gene into breast cancer samples carrying mutations in the PTEN gene. The experimental results showed that the breast cancer samples with PTEN gene mutation showed a stable proliferation trend of tumor cells over time versus the control group. Three lines of organoids (16PM0462, 16PM0408, and 8PM0050) that did not develop neoplasm, when the three TS genes were knocked out and 14PM0932 were further chosen for gene editing studies were used to investigate the effects of TS combinations. Then, tests were performed on organs that had triple (P53/PTEN/RB1) or quadruple (P53/PTEN/RB1/NF1) mutations. According to the experimental results, oncogenic KRAS individually is only sometimes mutated in breast cancer, but it can trigger carcinogenesis when overexpressed in primary breast cells. They also demonstrated that to transform normal breast epithelial cells, ablation of at least three TS genes is necessary. As demonstrated by their study, the ablation of a minimum of three tumor suppressor genes is required to

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transform normal breast epithelial cells. Experiments proposed that at least three TS genes must be rendered inactive for carcinogenesis to take place and that ER+ ductal breast cancer can be reproduced using CRISPR-Cas9 editing of TS genes [19]. Therefore, breast cancer may have the potential to be treated by reducing the ablation of TS genes.

In addition to the exploration of gene-specific mechanisms, CRISPR technology has also been applied to explore targets for coordinating therapeutic agents against breast cancer, such as MC1 and FGFR4. Cruz-Gordillo P et al. used a CRISPR-Cas9-mediated knockdown screen using the whole genome to identify a therapeutic targeting avenue that synergistically kills TNBC cells with EGFR inhibitors. Although TNBC and EGFR-mutated non-small cell lung cancer (NSCLC) cells share similarities in EGFR-dependent signaling and gene regulation, and both TNBC and NSCLC express high levels of EGFR signaling, TNBC is insensitive to EGFR inhibition, whereas NSCLC cells typically react to EGFR inhibitors. A genome-wide screen for single gene knockouts was utilized to identify the genes causing EGFR inhibition resistance in TNBC, the researchers discovered that the ELP complex played a key role in fostering EGFR resistance. Moreover, MCL1 was one of the most abundantly missing genes in the CRISPR screen, suggesting an essential function of MCL1 expression in modulating the sensitivity to EGFR in TNBC by the ELP complex. The findings suggest that the sensitivity of TNBC to EGFR inhibitors can be enhanced by regulating the expression levels of the ELP complex and MCL1. This finding suggests that it may be possible to enhance the unexpected targeting of TNBC subtypes through the inhibition of MCL1 expression [20].

Combining in vitro and in vivo models of CRISPR screening with the known anti-HER2 target gene FGFR4.Researchers used the dCas9-KRAB-CRISPRi technology to decrease FGFR4 levels in anti-HER2 resistant cells in in vitro tests to further probe the action of FGFR4 about promoting HER2-targeted therapy resistance. The findings demonstrated that trastuzumab resistance, an anti-HER2 breast cancer therapeutic agent, was greatly ameliorated after inhibition of FGFR4 in cell lines with endogenous or acquired (rSKBR3) resistance to the drug. Overexpression of FGFR4 decreased the sensitivity of SKBR3 cell cultures to TDM-1 treatment, whereas FGFR4 inhibition heighten the sensitivity of cell lines to the drug [16].

The super-enhancers (SEs), are vast Enhancer clusters that drive specialized expression programs that determine cellulate homogeneity and play a major part in increasing the expression of cancer-driver genes, can likewise be subject to the knockout function of the CRISPR system [21,22]. MET is another oncogene that exhibits a strong expression in TNBC and is related to worse clinical results. high expression of ANLN as an oncogene in patients with breast carcinomatosis is significantly associated with poor patient survival.

Huang H et al. used transcriptomic and epigenetic analysis to identify TNBC-specific super-enhancers (SEs) and their target genes (FOXC1, MET, and ANLN) for their study. The investigators depend on CRISPR-Cas9 to silence two of the five enhancers (e1, e2) within the SSE245 region in three TNBC lines. Deletion of those enhancers resulted in a substantial reduction in FOXC1 expression in all TNBC lines and impairment of spheroid cell growth and cloning, consistent with its oncogenicity. And FOXC1 overexpression was able to reverse the FOXC1-SE deletion's suppression of spheroid development and invasiveness. This demonstrates that FOXC1 is the interest gene of FOXC1 SE and is engaged in tumor development in TNBC by SE regulation. Like FOXC1, the elimination of MET gene-associated SEs by CRISPR-Cas9 resulted in reduced colony formation, suppressed spheroid growth, and reduced cell invasiveness. The function of the ANLN gene in TNBC cloning was also demonstrated by the experiments of Huang H. CRSIRPR-mediated deletion of ANLN SEs resulted in a significant reduction in ANLN expression levels and clonogenicity. Thus FOXC1 and MET ANLN are all SE-regulated cancer factors and can affect the progression of breast cancer by different levels of SE knockout on the three genes [23].

4. Conclusions

With high cure rates for early-stage breast cancer, there has been a significantly improved in the therapy and prevention of breast cancer. However, mid to advanced breast cancer remains incurable and still

poses a major challenge to patients. To address this issue, this paper summarizes some recent in vivo CRISPR screens or experiments of genes in different allogeneic models to find new regulators and targets of breast cancer immunity and finds that the CRISPR/Cas9 system is advantageous in probing tumorigenic and response mechanisms to help people better develop novel targeted drugs or improve the efficiency of approved drugs in patient treatment species. The CRISPR/ Cas9 system has proven to be a breakthrough instrument for treating a variety of diseases including cancer because of its simplicity, efficiency, specificity, accuracy, and persistence in genome editing. Although the CRISPR system's usage in humans creates several social and moral dilemmas, as biological and environmental aspects, in laboratory and animal models applications of CRISPR technology mentioned in this paper demonstrate the feasibility of using CRISPR to investigate the etiology of breast cancer and find therapeutic targets, which holds great promise for the remedy of breast cancer. Despite challenges that remain with CRISPR technology such as off-target effects and delivery vectors, researchers are overcoming these obstacles through various approaches to pave the way for the exploitation of CRISPR systems to research cancer mechanisms and develop cancer drugs.

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