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Construction of the pEGFP-N1-p53/MAR Vector and Its Effect on HEP3B Cell Morphology

(Pembinaan Vektor pEGFP-N1-p53/MAR dan Kesannya ke atas Morfologi Sel HEP3B)

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ABSTRACT

Cancer always presents a big problem that endangers human health. In recent years, the use of gene therapy in cancer research has significantly increased. This study aimed to construct a non-viral, wild-type, recombinant eukaryotic expression vector, pEGFP-N1-p53/MAR and verify its mechanism of action in cancer cells in vitro. *This investigation provides a novel strategy for p53 gene therapy via regulation of the matrix attachment region (MAR), potentially laying a foundation for the establishment of an anticancer protein bioreactor. The p53 gene was cloned from human peripheral blood and the MAR gene was amplified from chicken liver tissue. The recombinant eukaryotic expression vector pEGFP-N1-p53/MAR was constructed using an* E. coli *self-replication system. Lipofectamine™ 2000 was used as the transfection agent to deliver the plasmid into the human hepatic carcinoma (HEP3B) cell line. We divided the groups as follows: negative control cells without plasmid transfection, vehicle control cells transfected with the PEGFP-N1 vector, and experimental cells transfected with the pEGFP-N1-p53/MAR vector. Cells in each well of the vehicle control and experimental groups were transfected with 1.6 μg of plasmid and 3 μL of liposome. The cellular morphology of each group was analysed using green fluorescence microscopy at 12, 24, 36 and 48 h. Then, statistical analysis of the apoptosis rates among the three groups was performed using SPSS. The ultrastructures of the cells were observed via transmission electron microscopy after transfection for 24 h. Morphological analysis showed that the cells of the experimental group were shrunken and reduced in size and their intercellular connections had disappeared. Additionally, the apoptosis rate in the experimental group was significantly higher than that in the control groups and the cellular microstructure showed that heterochromatin and apoptotic bodies were found in the experimental group. In conclusion, compared with the control groups, the pEGFP-N1-p53/MAR plasmid can effectively promote Hep3B cell apoptosis* in vitro.

Keywords: Cell microstructure; cell morphology; gene therapy; p53 gene; matrix attachment region (MAR); non-viral vector

ABSTRAK

Kanser selalu menimbulkan masalah besar yang mengancam kesihatan manusia. Kebelakangan ini, penggunaan terapi gen dalam penyelidikan kanser telah meningkat dengan ketara. Kajian ini bertujuan untuk membina sebuah rekombinan vektor ekspresi eukariot bukan virus, jenis liar, pEGFP-N1-p53/MAR dan mengesahkan mekanisme tindakan dalam sel kanser secara in vitro. *Kajian ini memberikan satu strategi baru untuk terapi gen p53 melalui peraturan matriks pelekatan rantau (MAR), berpotensi meletakkan asas yang kukuh untuk penubuhan bioreaktor protein antikanser. Gen p53 diklon daripada darah periferi manusia dan gen MAR telah diamplifikasi daripada tisu hati ayam. Vektor ekspresi rekombinan eukariot pEGFP-N1-p53/MAR telah dibangunkan menggunakan sistem replikasi sendiri* E. coli. *Lipofectamine™ 2000 telah digunakan sebagai ejen transfeksi untuk menghantar plasmid ke dalam titisan sel manusia karsinoma hepar (HEP3B). Kami membahagikan kumpulan seperti berikut: Sel kawalan negatif tanpa transfeksi plasmid, sel kawalan penghantaran transfeksi dengan vektor PEGFP-N1 dan sel uji kaji transfeksi dengan vektor pEGFP-N1-p53/MAR. Sel dalam telaga setiap kawalan sarana dan kumpulan uji kaji telah ditransfeksi dengan 1.6 μg plasmid dan 3 μL liposom. Morfologi sel setiap kumpulan dianalisis menggunakan mikroskop hijau pendarfluor pada 12, 24, 36 dan 48 jam. Kemudian, analisis statistik pada kadar apoptosis antara ketiga-tiga kumpulan telah dijalankan menggunakan perisian SPSS. Ultrastruktur sel telah diperhatikan melalui penghantaran elektron mikroskop selepas transfeksi selama 24 jam. Analisis morfologi menunjukkan bahawa sel kumpulan uji kaji telah dikecut dan dikurangkan saiznya serta sambungan intersel telah hilang. Di samping itu, kadar apoptosis dalam kumpulan uji kaji adalah jauh lebih tinggi daripada kumpulan kawalan dan mikrostruktur sel menunjukkan bahawa jasad heterokromatin dan apoptotik telah ditemui dalam kumpulan uji kaji. Kesimpulannya, berbanding dengan kumpulan kawalan, plasmid pEGFP-N1-p53/MAR berkesan menggalakkan sel apoptosis Hep3B secara* in vitro.

Kata kunci: Gen p53; matriks pelekatan rantau (MAR); mikrostruktur sel; morfologi sel; terapi gen; vektor bukan virus

Introduction

Gene therapy can be defined as the transfer of foreign genetic materials to a patient to prevent, treat, or cure a disease (Wang et al. 2015). Recently, there has been a significant increase in the development of gene therapies for the treatment of cancer, such as the transfer of suicide genes and tumour suppressor genes (Seth 2005). The p53 gene is the tumour suppressor gene most often associated with human tumours. More than 50% of cancers are associated with deletion and mutation of the p53 gene, which has now become one of the most important targets for cancer treatment (Buyukpinarbasili et al. 2016). The development of both viral and non-viral gene delivery methods is necessary to help therapeutic genes enter cancer cells. A high transfection efficiency of viral vectors has been reported; however, they have the potential to be mutagenic or immunogenic, and safety concerns have significantly restricted their clinical applications (Choi et al. 2012; Hafner et al. 2013; Naim 2013). Although the development of non-viral systems has suffered from a low gene-transfer efficacy, in some cases, their improved safety and specificity, greater flexibility and ease of manufacturing have rendered them attractive candidates for gene therapy (Xu et al. 2011). Therefore, in this article, we aim to construct a non-viral p53 vector with a high expression efficiency.

Active chromatin anchored to the nuclear matrix by a matrix attachment region (MAR) is limited to free rotation, which can lead to a torsion effect on the chromatin structure domain. This is a characteristic of MAR that allows for its random integration on the chromosome (Bode et al. 1992). Moreover, the single strand of MAR that adheres to the nuclear chromatin wire ring on the frame easily forms a superhelix and uncoils. As the degree of the superhelix increases, the uncoiling becomes less rigid. Meanwhile, these factors favour the combination of transcriptional regulatory factors and boost gene expression (Bode et al. 1992). MAR plays important roles, such as improving the expression level of genetically modified genes, eliminating gene expression differences in an individual and inhibiting the silence of exogenous genes. Transgenic studies have demonstrated that high-level tissue-specific expression is observed only when the core is present in the context of the MARs (Forrester et al. 1994). Overexpression of B cell regulator of immunoreactive growth hormone (IgH) transcription leads to enhanced DNase I sensitivity of endogenous immunoglobulin heavy-chain intronic enhancer matrix associating regions (Kaplan et al. 2001).

Based on the research described, we attempted to construct a new non-viral recombinant eukaryotic expression vector, pEGFP-N1-p53/MAR, for use in gene therapy and to build an anticancer protein bioreactor. We aimed to transfect HEP3B cells *in vitro* to verify the activity of the p53 gene in this recombinant vector, as this was the first time MAR was studied in the regulation of the p53 gene in HEP3B cells. Furthermore, the foreign gene was introduced into cells by Lipofectamine™ 2000. Liposomes have many advantages as gene delivery vehicles, such as a lack of immunogenicity and mutagenicity, the ability to complex with relatively large amounts of DNA, reliability in transfecting cells and ease of preparation (Wang et al. 2015). Enhanced green fluorescence protein was observed by green fluorescence microscopy. Subsequently, the selection of different groups with the same cell culture period was observed by transmission electron microscopy. Therefore, by comparing the microcellular morphological changes and internal changes in the ultrastructures of different groups of cells, we determined the inhibition efficacy of HEP3B cell growth after transfection with the recombinant expression vector.

Materials and Methods

Sample collection and test animals

The peripheral blood of early lung cancer patients was provided by the Third Affiliated Hospital Affiliated of Henan University of Science and Technology. The animal experimental design and procedures were approved by the Institutional Animal Care and Use Committee of Henan University of Science and Technology (Henan, People's Republic of China). The chickens were obtained from the genetics and breeding laboratory of the Animal Science and Technology College at Henan University of Science and Technology. Livers were collected from several healthy native Hy-Line Variety Brown hens.

RNA extraction and reverse transcription

Total RNA was extracted from the peripheral blood of early lung cancer patients using Trizol reagent (Takara, Beijing, China). The RNA purity and concentration were determined by agarose gel imaging systems. The RNA was then stored in liquid nitrogen. cDNA was synthesized by reverse transcription using the RT kit (Takara, Beijing, China).

Genomic DNA extraction

Genomic DNA was extracted from fresh chicken livers using the animal genome DNA extraction kit (Sangon, Shanghai, China). The DNA concentration and purity were determined using an agarose gel imaging system. The DNA was then stored at -20°C.

Primer design and synthesis

The p53 gene primers were designed according to the human tumour suppressor gene p53 sequence (NCBI GenBank: NW_926584.1) with HindIII and BamHI restriction sites (Table 1). Amplification of the p53 gene sequence was performed using PCR and the size of the p53 gene fragment was 1183 bp. MAR primers were designed based on the chicken α-globin MAR sequence (NCBI GenBank: AF098919.2) with XbaI and NotI restriction

sites (Table 1), producing a PCR product that was 1044 bp in size. All the primers were designed using Primer 5.0 and synthesized by Shanghai Sangon Biological CO., Ltd.

PCR AMPLIFICATION AND RECOVERY OF TARGET GENES

The PCR amplification reaction system included 10 μL of 2×Taq PCR Master Mix, 0.6 μL of forward primer, 0.6 μL of reverse primer, 1 μL of template and RNase-Free dH2O in a total volume of 25 μL. The cDNA of human peripheral blood was used as the template to amplify the p53 gene. The MAR gene was amplified using the genomic DNA of the chicken liver as a template. Amplification was performed as described in Table 2. The conditions consisted of 1 cycle of pre-degeneration and final extension, followed by 32 cycles of denaturation, annealing and extension. PCR products were separated by 0.8% agarose gel electrophoresis, purified using a purification kit (Sangon, Shanghai, China) and sequenced by Shanghai Sangon Biological CO., Ltd.

CONSTRUCTION OF THE RECOMBINANT VECTOR

The pEGFP-N1 vector and p53 gene sequence were double-digested with HindIII and BamHI simultaneously and then ligated overnight with T4 DNA ligase at 4°. The self-replicating system of *Escherichia coli* was used to construct the pEGFP-N1-p53 vector. Both the pEGFP-N1-p53 vector and MAR were double-digested with NotI and XbaI simultaneously and ligated by the T4 DNA ligase to construct the pEGFP-N1-p53/MAR eukaryotic expression vector. Then, the recombinant vector PEGFP-N1-p53/MAR was transformed into *E. coli JM110*. Positive clones were screened by PCR and identified by sequence analysis. The recombinant vector was extracted from a transformant using the Mini Plasmid kit (Trans, Beijing, China).

CULTURE AND TRANSFECTION OF HEP3B CELLS

HEP3B cells were obtained from the Institute of Basic Medical Science at the Chinese Academy of Medical Science. The cell lines were characterized by the provider using gene profiling analysis and used fewer than 6 months after receipt. HEP3B cells were cultured in Minimum Essential Medium (HyClone, USA) supplemented with 10% foetal bovine serum(v/v) and 1% penicillin/streptomycin (v/v) at 37°C in a humidified atmosphere containing 5% CO_2. Then, 1×10^5 cells were plated on 12-well plates. Prior to the transfection experiments, the cells were plated at 80% confluence per well in twelve-well plates. The Hep3b cells were transfected with plasmids using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells in each well of the vehicle control and experimental group were transfected with 1.6 μg of plasmid and 3 μL of liposome. Then, the cells were divided into three groups as follows: PEGFP-N1-p53/MAR, PEGFP-N1 and the normal group without any plasmids, which represent the experimental group, the vehicle control group and the negative control group, respectively.

DETECTION OF MORPHOLOGICAL CHANGES IN HEP3B CELLS

Both the PEGFP-N1-p53/MAR and PEGFP-N1 vectors carry the GFP gene; thus, the transfection efficiency could be evaluated by GFP expression. Green fluorescence indicated by expression of the GFP gene was observed every 12 h after transfection under a fluorescence microscope (CX41, Olympus, Tokyo, Japan) for every group. Simultaneously, morphological changes in the cells were observed every 12 h (12 h, 24 h, 36 h, 48 h) after transfection using an inverted microscope (Nikon 2000-U; Japan). After transfection for 48 h, apoptosis was detected using a TUNEL Kit (Roche, USA) and statistical analysis of the apoptosis rates among the three groups was performed using SPSS 20.0 software.

TRANSMISSION ELECTRON MICROSCOPY (TEM) ASSAY

The transfected cells were collected at 24 h. The collected cells were washed three times in 0.1 M sodium phosphate buffer at pH7.4 and fixed in 4% glutaraldehyde for 48 h at 4°. The fixed cells were washed three times in 0.1 M sodium phosphate buffer for 5 min each and post-fixed in 1% OsO4 and 0.1 M sodium phosphate buffer for two h

TABLE 1. Primer sequences for PCR

	Primers	Size/bp
P53	For: 5'-CGCaagcttATGGAGGAGCCGCAGTC-3' Re: 5'-GCCggatccCAGTCTGAATCAGGCCCT-3'	1183
MAR	For: 5'-TATAgcggccgcCACTGTAGCCCTTA-3' Re: 5'-CTACtctagaGCTGGAAATGGCAAAC-3'	1044

TABLE 2. PCR amplification condition for p53 and MAR genes

	Pre-degeneration °C/min	Denaturation °C/s	Annealing °C/s	Extention °C/s	Final extention °C/min
P53	95/4	94/30	60/40	72/40	72/10
MAR	94/5	94/30	59/30	72/30	72/10

at 4°. The samples were dehydrated in a gradient ethanol series (30%, 50%, 70%, 90% and 100%) and embedded in Epon with different labels. Ultrathin sections were cut at 60 nm using LKB NOVA and examined under a Tecai G2 Spirit transmission electron microscope. The morphological characteristics of the transfected cells were compared among the three groups.

Results and Disscussion

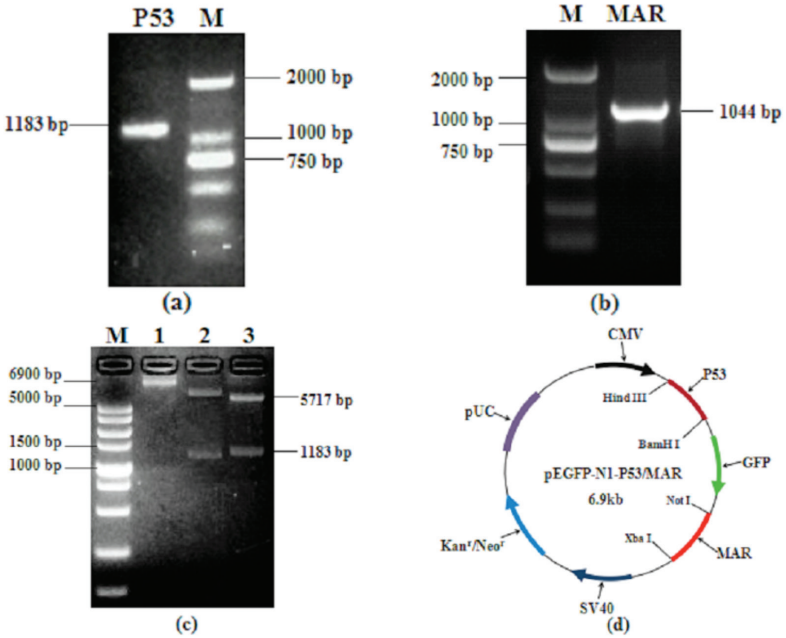
Successful construction of the pEGFP-N1-p53/MAR vector

The human tumour suppressor gene p53 sequence was amplified by RT-PCR and a single band was obtained by 1% agarose gel electrophoresis (Figure 1(a)). The sequencing comparison with known sequences (GenBank: NW_926584.1) resulted in 99% homology. Using chicken MAR gene primers, the gene fragment of chicken MAR was amplified by PCR, and a single band was obtained by 1% agarose gel electrophoresis (Figure 1(b)). The BLAST result showed 99% homology to the NCBI sequence (GenBank: AF098919.2). Subsequently, the purified recombinant plasmid DNA pEGFP-N1-p53/MAR was separated by 1% agarose gel electrophoresis and detected by UV light. The plasmid was identified by enzyme digestion (Figure 1(c)) and sequenced with universal primers. The recombinant eukaryotic expression vector pEGFP-N1-p53/MAR was successfully constructed. The recombinant expression vector pEGFP-N1-p53/MAR included the human tumour suppressor gene p53 and the chicken MAR gene regulatory sequence. In addition, the p53 sequence was inserted between HindIII and BamHI and the locus of the MAR sequence was inserted between NotI and XbaI (Figure 1(d)).

The molecular weight of the protein encoded by the p53 gene is approximately 53 KD, which is why it is called the p53 protein. It is composed of 393 amino acid residues and can be detectable in nearly all somatic cells (Soussi et al. 1996, 1990). The principle function of p53 protein is to integrate the stress response of cells to crisis situations, which relays messages that influence the structure and function of DNA stability (Levine et al. 1997). The biological function of the normal p53 protein is two-faced in the lives of healthy cells. It acts on the damaged cells in G1 and restores the DNA of damaged cells before their duplication and fission. However, when cell damage occurs beyond the extent to which the p53 gene can repair, this gene induces cell apoptosis (Ljungman 2000; Soussi 2007). Treatments have investigated p53 gene therapy in combination with radiotherapy, physical therapy, chemotherapy and gene therapy targeting other genes (Liu et al. 2010; Mandal et al. 2011; Ndoye et al. 2004; Xu et al. 2002).

Macroscopic morphology changes in Hep3B cells

Morphological changes in the transfected HEP3B cells in the three groups over time are presented in Figure 2(a).



(a) Lane M indicates DNA Mark 2000; Lane p53 indicates the p53 gene sequence, (b) Lane M indicates DNA Mark 2000; Lane MAR indicates the MAR gene sequence, (c) Lane M indicates DNA Mark 5000; Lane 1 indicates the plasmid DNA pEGFP-N1-p53 digested with HindIII (only one line: 6900 bp); Lane 2 indicates the plasmid DNA pEGFP-N1-p53/MAR double digested with NotI and XbaI (two lines: 5856 bp, 1044 bp); Lane 3 indicates the plasmid DNA pEGFP-N1-p53/MAR digested with HindIII and BamHI (two lines: 5717 bp, 1183 bp) and (d) The pattern diagram of the recombinant expression vector pEGFP-N1-p53/MAR

FIGURE 1. Diagram of the recombinant plasmid pEGFP-N1-p53/MAR

The transfected cells in the negative control group and vehicle control group showed normal structural features, as indicated by the adherent morphology and integrity of the cell junctions (Figure 2(a)-N/P-12～48 h). However, as the experiment continued, the morphology of the experimental group showed not only cell shrinkage and cell size reduction but also the disappearance of intercellular connections (Figure 2(a)-E-12～48 h). Many cells died in the experimental group at 48 h after transfection (Figure 2(a)-E-48 h).

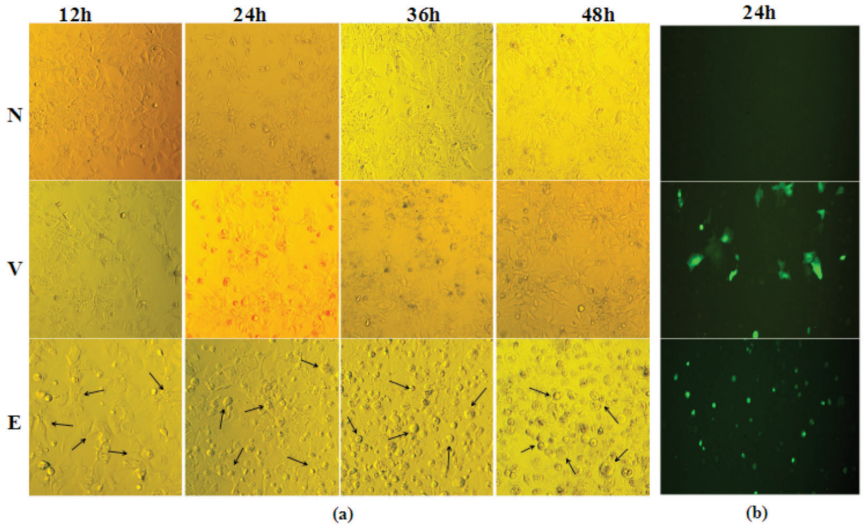
Expression of the GFP protein was observed in both the vehicle control and experimental groups of HEP3B cells. HEP3B cells in the vehicle control and experimental groups showed green fluorescence after transfection (Figure 2(b)-P/E-24). Cells in the negative control group never showed any fluorescence (Figure 2(b)-N-24 h).

HEP3B cells are a human hepatoma cell line that has a non-functional p53 gene (Cheng et al. 2010). The transfection of the recombinant carrier pEGFP-N1-p53/MAR to HEP3B cells excludes the possibility of the HEP3B cells producing the p53 protein. The reporter gene EGFP in the pEGFP-N1-p53/MAR carrier can be used to detect the transfection efficiency of the foreign gene under fluorescence microscopy (Figure 2(b)). After the three groups of cells were transfected, the morphology of the cells in the negative control and vehicle control groups changed minimally over time, and the cells adhered well (Figure 2(a)-N/P). By contrast, the experimental group was transfected with the recombinant plasmid pEGFP-N1-p53/MAR, which clearly changed the cell morphology (Figure 2(a)-E). After transfection for 12 h, the cells of the experimental group began to shrink and an increasing number of cells became clearly crinkled and rounded from 24 h to 36 h. Morphological studies have shown that typical apoptotic cell shrink and their cytoplasm condenses (Hacker 2000; Majno & Joris 1995). Large amounts of HEP3B cells were apoptotic at 48 h compared to that in the control groups when analysing the apoptosis rate (Figure 4). The results of this study are consistent with research on the wild-type p53 expression product, which can inhibit the growth of cancer and induce cell apoptosis (Ko & Prives 1996). This study shows that the recombinant eukaryotic expression vector pEGFP-N1-p53/MAR can effectively inhibit the growth of HEP3B cells, which could be mediated by the expression of p53 in cells.

CHANGES IN THE UITRASTRUCTURE OF HEB3P CELLS

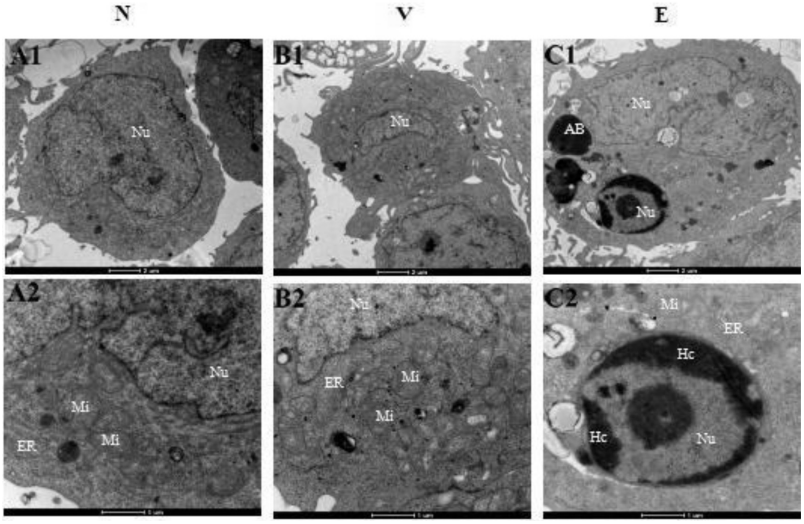
As shown in Figure 3, the ultrastructural changes of the three groups were analysed at 24 h after transfection, showing different features among the groups. Ultrastructural analysis of the negative control (Figure 3-A1/A2) and vehicle control (Figure 3-B1/B2) groups showed normal rough endoplasmic reticulum, mitochondria and nuclear structures. After transfection with pEGFP-N1-p53/MAR, however, the cells presented severe apoptotic phenomena (Figure 3-C1/C2). Rough endoplasmic reticulum dilation and mitochondrial swelling appeared in the experimental group. Furthermore, heterochromatin and apoptotic bodies appeared and a crescent was formed in the nucleus.

Transmission electron microscopy can observe cell structure changes at different stages of apoptosis, which is considered to be the classic method of cell apoptosis research (Kerr et al. 1972). The ultrastructures of the HEP3B cells were observed by transmission electron microscopy 24 h after transfection. In the vehicle control group, the mitochondria were slightly swelled compared with those of the negative control group. This finding was due to the slight toxicity of green fluorescent protein to HEP3B cells. After observing the nuclear structure,



(a) Morphological changes in the transfected Hep3b cells among the three groups over time (100×). Arrows point to the part of the cell morphology that change over time and (b) Observation of green fluorescence in the Hep3b cells of the three groups at 24 h after transfection under fluorescence microscopy at a wavelength of 480 nm (Note: N, negative control group; V, vehicle control group; E, experimental group)

FIGURE 2. Diagram of transfected Hep3B cells in the three groups

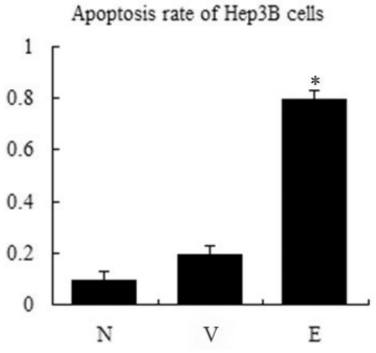


All cells were sliced after transfection for 24 h. The pictures of A1, B1 and C1 were obtained at 6000× magnification. The pictures of A2, B2 and C2 were obtained at 12000× magnification (Note: N, negative control group; V, vehicle control group; E, experimental group; Nu, nucleus; Mi, mitochondria; ER, endoplasmic reticulum; Hc, heterochromatin; AB, apoptotic body)

FIGURE 3. Ultrastructure of transfected Hep3b cells under transmission electron microscopy

heterochromatin was not found in the two groups (Figure 3-A2/B2). Cells in the vehicle control had smaller nuclei than those in the negative group (Figure 3-A1/B1). This result may have been due to the slice angle problem during cell sectioning. In addition, the nuclei of the tumour cells were irregular and some cells showed multicellular nuclei. Therefore, the nuclear structure was smaller and did not directly show the difference in the cell structure between the two groups. Heterochromatin was found in the cells of the experimental group and was concentrated on the edge of the nucleus with a typical crescent shape (Figure 3-C2). Meanwhile, apoptotic bodies were found, the mitochondria were swollen and the content of the rough endoplasmic reticulum was reduced (Figure 3-C1/C2). The ultrastructural morphological characteristics of hepatocyte apoptosis include nuclear retraction or fragmentation, cell membrane integrity and the formation of apoptotic bodies (Saafi et al. 2001). The changes in the ultrastructure of the experimental group cells fully demonstrate that transfection of the recombinant expression plasmid pEGFP-N1-p53/MAR can promote the apoptosis of HEP3B cells.

SPSS (Statistic Package for Social Science) analysis of the apoptosis rates among the three groups are shown in Figure 4. The apoptosis rate of the experimental group was significantly higher than those of the negative control group and the positive control group ($p<0.05$). There was no significant difference between the negative and positive control groups. All the results of this study have proven that in vitro transfection of the pEGFP-N1-p53/MAR vector can effectively promote Hep3B cell apoptosis.



The level of cell apoptosis in each group was recorded by the TUNEL method after 48 h of transfection. Three repetitions in each group were analysed by SPSS single factor analysis of variance (Note: * $P<0.05$, significant difference; N, negative control group; V, vehicle control group; E, experimental group)

FIGURE 4. Statistical chart of the apoptosis rates of Hep3B cells

CONCLUSION

In this study, we successfully constructed a recombinant eukaryotic expression vector with the wild-type p53 gene and MAR regulation sequence. This study showed that transfection of the pEGFP-N1-p53/MAR vector can inhibit the proliferation of HEP3B cells, which can be mediated by the expression of p53 in cells. However, future studies on the expression of p53 and the role of MAR in conferring anticancer properties should be performed, as they will provide new research and exploration regarding p53 gene therapy. Meanwhile, the successful construction of the recombinant eukaryotic expression vector pEGFP-N1-p53/MAR also lays a foundation for the establishment of an anticancer protein bioreactor.

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