

Original Research

View Article Online



Received 18 November 2021

Revised 01 December 2021

Accepted 02 December 2021

Available online 06 January 2022

Edited by Barbara Sawicka

KEYWORDS:

Solanum tuberosum
 β -glucuronidase
Paulownia tomentosa
Chitosan nanoparticle
UidA gene

Natr Resour Human Health 2022; 2 (2): 215-221
<https://doi.org/10.53365/nrhh/144414>
ISSN: 2583-1194
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Chitosan nanoparticles as a new technique in gene transformation into different plants tissues

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ABSTRACT: The utilization of chitosan nanoparticles is a novel technique for gene transformation into plant tissues. It takes a few minutes to transform gene to plant. UidA gene was detected in *Escherichia coli* (K12 strain) using polymerase chain reaction analysis by UidA-specific primers. The gene was transformed into the explants of two different plant species (*Solanum tuberosum* and *Paulownia tomentosa*). These plants have different natures as crop and woody plants respectively. Therefore, they have different abilities to express the UidA gene. The gene is expressed into blue color in plant tissues due to the formation and expression of the GUS enzyme. The transformation of the UidA gene was detected morphologically by the formation of blue color; and molecular using PCR. Chitosan nanoparticles were characterized by UV/Visible spectroscope and photographing with a transmission electron microscope (TEM). As a result of this research, it is suggested that chitosan nanoparticles be used in gene transformation into plant tissues. Because it is safe, quick, and inexpensive, as well as biocompatible and biodegradable.

1. INTRODUCTION

UidA gene was obtained from *Escherichia coli* (K12 strain) and codes for β -glucuronidase enzyme which is of hydrolase type. This enzyme cleaves a variety of glucuronides which is category of glycosides. In higher plants there is nearly no detection for activity of GUS (β -glucuronidase) in normal conditions. The gus's substrates are used for histochemical, fluorometric and spectrometric essays due to its visible color (Ruijter et al., 2003). Glycosyl hydrolases are an important category of enzymes which hydrolyze the glycosidic bond in synthesis of carbohydrates. β -glucuronidase enzyme is a sort of glycosyl hydrolase that hydrolyze β -glucuronic acid remains from the non-reducing ends of glycosaminoglycans (GAGs). In prokaryotes, the sequence of *E. coli* β -glucuronidase gene (UidA) encoded a stable enzyme. GUS gene (UidA) developed within *E. coli* as a marker for analyzing gene expression in transformation into plant tissue. The mechanism of resulted GUS enzyme acts by splitting of the β -glucuronic acid from the substrate X-glass and this forms a blue color in transforming tissues. This expression stems from the sensitivity and stability of the reporter essay. Transgenic plants tissues expressing GUS display blue color in histochemical staining (GUS assay) due to the substrate "5-Bromo-4-chloro-3-indolyl-beta- D-glucuronide (X-Gluc)" (Arul et al., 2008).

Chitosan is a natural chemical that aids in various anti-obesity treatments and is highly recommended for weight loss. It has a variety of useful features, including non-toxicity, biocompatibility, biodegradability, antibacterial activity, chemical reactivity, industrial uses, and body fat carrier, industrial applications (Cummings et al., 2010; Hennen, 2005).

DNA transformation was performed by Bruce (2002) who explained a cell's genetic alteration caused by the direct uptake of foreign DNA from its environment across the cell membrane. This method is applied recently by Hussien (2020) who mentioned that efficient transformation was performed by loading the target gene directly on the surface of chitosan nanoparticles.

Chitosan nanoparticle is a method to transform and insert genes into different tissues. G. Li et al. (2015) mentioned that chitosan is a non-viral vector, natural biodegradable cationic alkaline polysaccharide, and stable cationic polymer. Chitosan (CS) binds strongly to negatively charged DNA due to its positive amino group. Also, Riva et al. (2011) also listed the properties of CS, which include its biodegradability, nontoxicity, biocompatible, semi-crystalline, linear polysaccharide, able to heal wounds, and low cost. It's a polysaccharide made up of "-(1-4)-linked D-Glucosamine" (deacetylated unit) and "N-acetyl-D-Glucosamine" (N-acetyl-D-Glucosamine) (acetylated unit). Cabuk et al. (2015) mentioned that there are two impor-

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tant mechanisms of chitosan/DNA binding: (1) Electrostatic Interaction and cell uptake by direct mixing of the positive chitosan charge with the negative nucleic acid charge. (2) Polymer-based vectors can penetrate cells via the interaction of charge-mediated with negatively charged proteoglycans on the cell membrane structure or endocytosis.

Chitosan nanoparticles or complexes that safeguard genetic material for cellular internalization must have appropriate stabilities that not only successfully protect genes but also allow gene release inside the cell following uptake. Chitosan particles or complexes influence cellular uptake and release of gene inside cytoplasm, and finally influence transfection efficacy. To begin with, chitosan MW has an impact on the creation of nanoparticles or polyplexes (Cao et al., 2019; Lee & Mohapatra, 2008; Ragelle et al., 2013).

This survey reports for the achievement of applying chitosan nanoparticles as a novel, safe and fast method for optimization of transformation of *UidA* gene into plant tissues and regeneration of transgenic plantlets.

2. MATERIALS AND METHODS

2.1. Plant materials

Two different plant species were used for this transformation study. *Solanum tuberosum* as crop plant and *Paulownia tomentosa* as woody tree plant. They were cultured on free MS media (CAISSON) for multiplication.

2.2. Obtaining of *UidA* gene and Cloning

E. coli (K12) was grown in LB broth at 37 °C overnight at 200 rpm. The cells were harvested by centrifugation and DNA was extracted following the protocol of He (2011). The primers specific for *UidA* gene were designed by snap gene® (2.3.3) based on wide gene sequence identified in NCBI database (GenBank accession no: **EG11055**). The designed primers sequence was: (*uidAF*): 5-ATGTTACGTCCTGTAGAAACCC-3 and (*uidAR*): 3-TACATTGAGTGCAGCCCGGC-5. These primers were obtained from Bioline (Cat#BIO-41026). The PCR reaction of *UidA* gene was applied for 35 cycles including: “30 sec min at 94°C, 30 sec at 64°C and 50 sec at 72°C”. The PCR product was checked on 1.4% (w/v) agarose gel compared to a hyper ladder 1Kb (Bioline, Cat#BIO-33053). Then the insert was purified using “the GeneJET™ PCR Purification Kit (Cat. # Thermo K0701)”.

The ligation protocol was as follow: “The *UidA* insert was ligated into pMiniT vector (supplied from NEB® PCR Cloning Kit, #E1202S, supplemented by NEB 10-beta competent Cells (NEB #C3019)), then ligated to pMiniT according to the manual instruction. The antibiotic resistance gene is for “Ampicillin”, in concentration of 50 µg/ml; with constitutive promoter which is active in vivo in all conditions”.

2.3. Bacterial Transformation Confirmation

The non-recombinant and recombinant colonies were identified by colony PCR as follows: “The recombinant bacteria

were cultured on LB agar plates with 100 g/L ampicillin. A single colony from the bacterial transformation was used as a template for the confirmatory PCR reaction after growth. For this reaction, the primers and reaction conditions were the same as for the *UidA* gene detection in *E. coli* (K12)”. After that, the product was electrophoresed on 1.4% gel compared to 1 Kb ladder.

2.4. Chitosan Nanoparticle Transformation

2.4.1 Chitosan Nanoparticles Characterization

2.4.1.1 Chitosan Deacetylation Degree The degree of deacetylation (DD) of chitosan nanoparticles was determined using the titration method, as described by Czechowska-Biskup et al. (2012): “With constant stirring for 30 minutes, 0.2 g powder chitosan was dissolved in 25 cm³ of 0.1 M hydrochloric acid. After that, 25 mL of dH₂O was added, and the mixture was stirred for 30 minutes. The solution was titrated with a 0.1 moldm⁻³ sodium hydroxide solution once the chitosan was completely dissolved.” Degree of deacetylation (DA or DD) of chitosan was calculated using the formula:

$$DA\% = 2.03 \left(\frac{V2 - V1}{m + 0.0042 (V2 - V1)} \right)$$

Where m is the weight of the sample, V1 and V2 are the volumes of 0.1 moldm⁻³ sodium hydroxide solution corresponding to the deflection points, 2.03 is the coefficient resulting from the molecular weight of chitin monomer units, and 0.0042 is the coefficient resulting from the difference between the molecular weights of chitin and chitosan monomer units.

2.4.1.2 Transmission Electron Microscopy (TEM) The TEM methodology was followed by Kiang et al. at Al-Azhar University’s Regional Center for Mycology and Biotechnology (2004). “Carbon coated 400 mesh copper grids were placed on a drop of CS/pDNA complex, then for around 1.5 minutes,” according to the technique. The grid was stained for 1.5 minutes with a drop of 2 percent uranyl acetate filtered solution, with surplus uranyl acetate removed.” After drying for 10 minutes, the grids were inspected using a JOEL JEM.1010 transmission electron microscope.

2.4.1.3 UV-Visible Spectra Measurements The absorbance of chitosan nanoparticles was measured using UV-visible spectroscopy over a wide range of wavelengths. “JASCO V-630 UV-visible spectrophotometer (serial: C285061148)” was the instrument. Spectra Measurement was the additional software.

2.4.2 Chitosan-DNA Complex Formation (CS/pDNA)

According to Mansouri et al. (2006), the production of CS/DNA is as follows: “Dissolve chitosan in 25 mM acetic acid at a working concentration of 0.08 percent and adjust to pH 5.5.” For 15 minutes, the CS and recombinant pMiniT were incubated in a water bath at 55°C. The CS/pDNA combination was made by adding 0.08 percent CS to an equal volume

of pDNA solution (50 mg/ml) dissolved in 50 mM sodium sulphate and vortex mixing for 1 minute.”

2.4.3 DNA retention and release

X.W. Li et al. (2003) determined the binding between pDNA and CS on 1% agarose gel electrophoresis. While Huang et al. (2005) designed a DNA release from chitosan nanoparticles as follow: ”The pellet was re-suspended in equal volume of phosphate-buffered saline (PBS, pH 7.4) and incubated at 37°C with agitation at 100 rpm for 3 days after centrifugation at 4°C, 14000 g for 30 minutes.”

2.4.4 Chitosan/pDNA Transformation into Plant

Abdel-Razik et al. (2017) described the transfection of chitosan nanoparticles containing the recombinant plasmid into plant tissues as follows: ”the nodal segments of *Solanum* and *Paulownia* species were incubated for 40 minutes at room temperature in an eppendorf tube containing 400 l of CS/pDNA mix.” The explants were then transferred to MS media with 2 mg/l BA, 1 mg/l kin, and 100 g/L ampicillin, and cultured at 251 °C for 4 weeks to establish new transgenic lines of these species.”

2.5. Molecular Analysis of Transgenic Lines

The ”cetyl-trimethyl ammonium bromide (CTAB) method” was used to extract complete genomic DNA from the upper leaves of transgenic and non-transgenic *Solanum* and *Paulownia* lines (Rogers & Bendich, 1985). Then, using the same pair of primers used for the initial gene detection, DNA fragments of UidA transgenes were produced using PCR.

3. RESULTS

In this survey, the chitosan nanoparticles transformation was applied for UidA gene transformation into two different plant species (*S. tuberosum* and *P. tomentosa*). The applied tissue culture technique was nodal cutting technique (micropropagation). The molecular weight of the target UidA gene was 1812 bp.

3.1. Colony PCR

To confirm transformation into bacterial cells, colony PCR was performed using selected transformed bacterial colonies grown on LB medium supplemented with 100 µg/L ampicillin. The results obtained showed bands in 1812 bp and this indication for recombinant colonies (Figure 1). pMiniT is a compact bacterial vector that employs a toxic minigene for high efficiency cloning and subsequent in vitro transcription of PCR products. It contains SP6 constitutive promoter (for bacteriophage SP6 RNA polymerase) (Figure 2).

3.2. Chitosan nanoparticle transformation

3.2.1 Chitosan nanoparticle characterization

The chitosan nanoparticle was found as finely powdered particles with medium molecular weight (501 KDa), pH

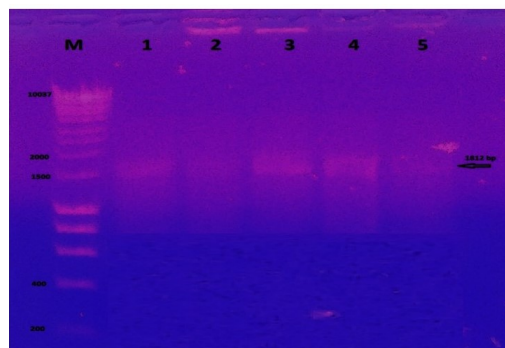


Figure 1. Agarose gel electrophoresis of colony PCR from competent 10-beta for the transformed UidA gene. Each lane indicates different colonies used for amplification of gene compared to 1 kb ladder.

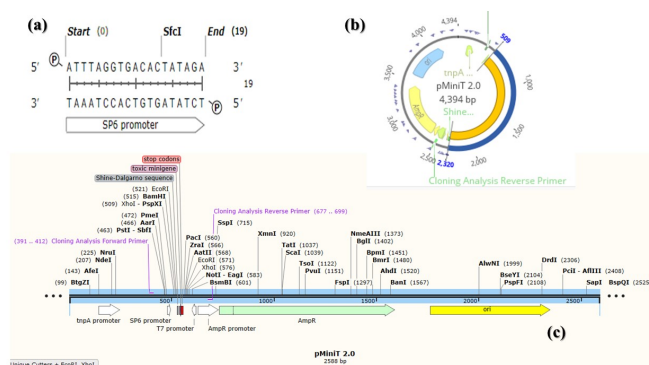


Figure 2. pMiniT vector data. (a) SP6 promoter for bacteriophage SP6 RNA polymerase, obtained by SnapGene (5.3.1); (b) Circular map of pMiniT showing promoters, cloning site and AmpR gene with the UidA insert (blue color), designed by Geneious Prime (2021.1.1); (c) Linear map of pMiniT vector designed by SnapGene (5.3.1).

4.4, average DD is $51.23 \pm 5.765\%$ and average particle size under transmission electron microscope was of 43.98 ± 1.24 nm (Figure 3). The UV-visible measurements of chitosan nanoparticles indicated the absorbance of chitosan nanoparticles at 302.8 nm (Figure 4).

3.2.2 DNA Retention and Release

Chitosan polymers with a positive charge were shown to have a high propensity to form complexes with DNA (-ve charge). According to agarose gel electrophoresis of complexes, the ideal complex formation ratio between CS and plasmid (pDNA) was 1:1. The molecular weight of CS is typically used to measure its capacity to encapsulate pDNA complexes. The findings demonstrated that the electrostatic interaction between CSNPs and pDNA, as well as charge neutralisation and/or an increase in the molecular size of the complexes, caused pDNA to be delayed from the well (Figure 5). The release of pDNA from CS-NPs as simulated in plant cells ensured that pDNA released from CSNPs and integrated into the chromosomal DNA of plant

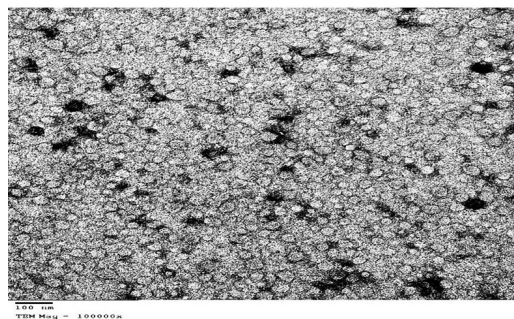


Figure 3. Transmission electron microscope image for CS/pDNA (chitosan nanoparticle binding with modified MiniT carrying UidA gene)

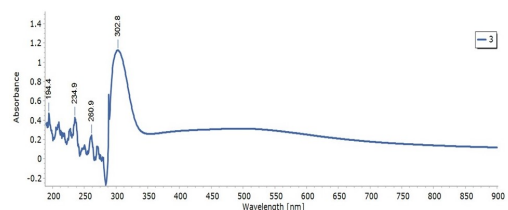


Figure 4. The UV-visible spectrum wavelengths of chitosan nanoparticles.

species (Figure 5).

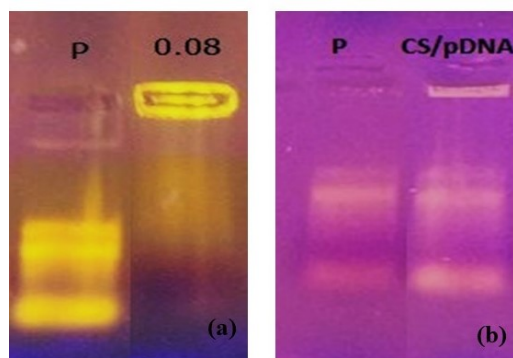


Figure 5. Agarose gel electrophoresis of (a) CS binding with modified pMiniT and (b) release pDNA from CS and both compared to control plasmid (without CS)

3.3. Confirmation of Transformation of CS/pDNA Into Transgenic Lines

The regenerated shoots are fully grown and multiplied after 4 weeks of incubation at 25°C (light conditions: 16 h light and

8 h dark). Then set of them was observed in the expression of blue color due to expression of the UidA gene and production of the GUS enzyme (Figure 6) as the stem of transgenic plant species is darker in color compared to non-transgenic. The other set of plants was examined for gene transfer using conventional PCR to detect the presence of the UidA gene in transgenic *S. tuberosum* and *P. tomentosa* compared to non-transgenic plants (Figure 7).



Figure 6. The transformed plant with UidA gene via CS (a) transgenic and non-transgenic *S. tuberosum* respectively in MS jars; (b) non-transgenic and transgenic *S. tuberosum* stems; (c) transgenic and non-transgenic *P. tomentosa* respectively in MS jars and (d) non-transgenic and transgenic *P. tomentosa* stems respectively.

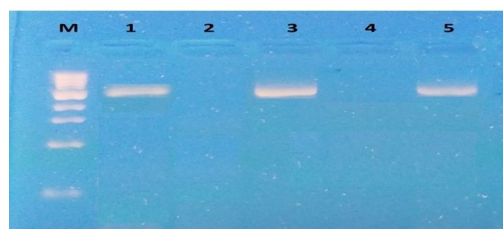


Figure 7. Gel electrophoresis of PCR products of the transformed plant species with UidA gene. M: 1 kb ladder; 1: UidA gene from *E. coli* (K12); 2: non-transgenic *S. tuberosum*; 3: transgenic *S. tuberosum*; 4: non-transgenic *P. tomentosa* and 5: transgenic *P. tomentosa*.

4. DISCUSSION

This study manipulated the transformation of UidA gene. This gene was isolated from *E. coli* (K12) as a prokaryotic organism and transformed into two different plant species (*S. tuberosum* and *P. tomentosa*) as eukaryotic organisms. This transformation occurred using chitosan nanoparticles.

The UidA gene also known as GUS gene and its expression leads to formation of blue color. The GUS gene is widely utilized

in plants because of its stability, simplicity, and resistance to a variety of chemicals and assay conditions such as pH and temperature. [Kavanagh et al. \(1988\)](#). After an extended period of storage, plants expressing GUS showed high levels of GUS activity. Many studies used UidA gene to be transformed into plant tissue, such as [Deng et al. \(2001\)](#) who transformed UidA gene into peanuts and [Mousavi et al. \(2014\)](#) who transformed the gene into Date plant.

CSNPs have many applications in pharmacy, agriculture, and gene delivery. CSNPs were used in transformation into different tissues like [Kiang et al. \(2004\)](#) who apply transformation of CS/pDNA carrying human embryonic kidney cell lines (HEK293); and [Hallaj-Nezhadi et al. \(2011\)](#) who loaded pUMVC3-hIL12 on CSNPs in Colon Carcinoma Cell lines. But there is a new trend to apply chitosan nanoparticles for transformation into plant tissues according to [Abdel-Razik et al. \(2017\)](#) and [Hussien \(2020\)](#). CSNPs are applied for gene transformation for the following reasons: cheap, biodegradable, biocompatible, nontoxic and save time compared to traditional *Agrobacterium* method. In *Agrobacterium* transformation, it requires co-cultivation, pre-selections for 3-5 days and requires antibiotics like cefotaxime to inhibit the action of *Agrobacterium*. Otherwise, using chitosan in transformation is faster as it only requires about half an hour to transform the plasmid into plant tissues, and it doesn't require antibiotics. Also, chitosan nanoparticle transformation efficiency is higher than that of *Agrobacterium* transformation. The size of chitosan/plasmid complexes was regulated by chitosan MW, according to [McLaughlin et al. \(1998\)](#) and [Kong et al. \(2012\)](#). "For example, 20, 40, and 80 kDa chitosan can create 200, 250, and 300 nm nanoparticles, respectively," they explained.

Many metal nanoparticles were applied in gene transformation like silver, gold, etc. For example, [Mortazavi and Zohrabi \(2018\)](#) used nano-gold to transform cry IA (c) gene into rice plant tissues. But it lacks the selectable marker gene. Besides, [Nagamani et al. \(2019\)](#) applied silver nanoparticles to enhance the transformation efficiency in *E. coli*. However, polymer nanoparticles like chitosan have safe effect, doesn't accumulated in cells and applicable for many transformation issues.

The complex of CS/pDNA was prepared with definite circumstances and applied an effective gene delivery system. The degree of deacetylation (DD) was estimated using simple titration method by [Czechowska-Biskup et al. \(2012\)](#) who determined "On the glycoside unit of CSNPs, the amine or acetyl amine groups (+ve) ligates with DNA" (-ve). Furthermore, the degree of deacetylation controls the amount of free amino groups in the polysaccharide, which play a key role in chitosan-DNA interaction." "DD of chitosan effects its solubility, crystallinity, and degradation," according to [Aiba \(1989\)](#). A higher DD indicates a higher positive charge, which allows for better DNA binding and cellular absorption." CSNPs were characterized because [Huang et al. \(2005\)](#) mentioned that these characteristics are essential in gene transformation for cellular uptakes of the CS/pDNA and its release from the endo-

lysosome pathway.

Chitosan has gotten a lot of attention as a polymeric carrier for delivering biomaterials into cells, specifically for delivering genes into mammalian and plant cells ([Bowman & Leong, 2006](#); [Hussien, 2020](#); [Thibault et al., 2010](#)). The polycationic polymer is a promising gene carrier with good gene transfer efficiency and a large loading capacity ([Mao et al., 2001](#)). Chitosan has previously been proven to mediate the opening of tight junctions for delivery reasons ([Abyadeh et al., 2017](#); [Grenha et al., 2007](#)). Interactions between chitosan and the cell membrane may potentially cause denaturation of membrane proteins and an increase in cell membrane permeability, according to the researchers.

The CS/pDNA ratio used in this study was 1:1. This indicated that the degree of deacetylation and molecular weight of CSNPs affect DNA binding with chitosan, and these findings were consistent with those of [Kiang et al. \(2004\)](#), who stated that "the ratio between CSNPs concentration and DNA must be equal." In addition, I concur with [Bivas-Benita et al. \(2004\)](#), who demonstrated that "particle size and shape play an important role in gene transformation into tissues." The release of CS/pDNA complex was performed and examined on agarose gel which agreed with [Bordelon et al. \(2011\)](#) who mentioned that the retardation in mobility of CS/pDNA on gel ensured complete encapsulation.

5. CONCLUSION

This work described a new streamlined approach for gene transformation using nanoparticles into plant tissue. Also, introduced chitosan nanoparticles (CSNPs) as a new technique in transformation instead of metal nanoparticles and traditional *Agrobacterium* protocol. All that due to the unique properties of CSNPs which is organic, biocompatible, biodegradable, and has high affinity to combine with DNA. UidA gene is a clear expressed gene and easily noticed due to the rapid formation of the blue color directly in the plant tissues. The transformation process was performed by direct transfer of the modified plasmid (cassette), loaded on chitosan nanoparticles, to the explant's segments. The incubation time allowed the gene to transform into plant tissues.

CONFLICTS OF INTEREST

All authors have nothing to disclose.

ACKNOWLEDGMENTS

The authors would like to thank Prof. Dr. Ashraf Bakry (Professor of genetics, Faculty of Agriculture, Ain Shams University) for providing us with chitosan nanoparticles. Also, thanks a lot for Agricultural center for genetic engineering and biotechnology "ACGEB", Faculty of Agriculture, Ain Shams University for facilitation of working in tissue culture labs.

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AUTHOR CONTRIBUTIONS

ET, MFA - Research concept and design, ET, MFA - Collection and/or assembly of data, ET - Data analysis and interpretation, ET - Writing the article, ET - Critical revision of the article, ET - Final approval of the article.

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