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Regular paper

Exploring the mechanism of miR320a in regulating PDL1 upon lung cancer pathogenesis

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The tumour suppressive role of miRNA320a is observed in many cancer types like in colon, lung, breast, and osteosarcoma but it is inversely reported in prostate cancer and in MPM cell lines. miRNA320a targets programmed death ligand 1 (PDL1) negatively in many cancer types and recently in Malignant pleural mesothelioma. In this background it is important to understand the regulatory mechanism of miRNA320a in determining PDL1 expression in different pathological stages of lung cancer. Histology was used to grade the initial and advanced stage of lung cancer following carcinogenic injection. Immunohistochemistry and Western blotting technique were used to analyse PDL1 protein expression. *In-situ* hybridization was used to determine miRNA320a signals. Initially, using the chemical carcinogen Diethylnitrosamine (25 µg/g), we successfully initiate initial and advanced stage of lung cancer following 6 months and 9 months of carcinogenic injection. The formation of initial and advanced stage of lung cancer is confirmed through histopathological changes which show neoplastic appearance in initial lung cancer and appearance of more mitotic cells along with tissue hardness in the advanced lung cancer stages. In miRNA320a blocked tissue the cancer condition becomes worse with decreased tissue elasticity along with more proliferative cells. Immunohistochemistry and Western blotting studies show that PDL1 is overexpressed in the advanced stages rather than in initial lung cancer because the expression of miRNA320a is overexpressed in initial stages but restricted in advanced stages of lung cancer. miRNA32a blocking studies confirm that miRNA320a expression act as a tumour suppressor that directly controls PDL1 expression that lack of miRNA320a enhances PDL1 expression as well as it triggers lung cancer advances. In summary, miRNA320a possess tumour suppressor function that limits PDL1 expression in initial lung cancer but its control over PDL1 suppression is lost once miRNA320a is downregulated in advanced stage of lung cancer.

Keyword: lung cancer, PDL1, miRNA320a, diethylnitrosamine, A/J mouse, histopathology

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Abbreviations: LNA, Locked Nucleic Acid; PDL1, programmed death ligand 1

INTRODUCTION

Lung cancer accounts for a leading cancer death worldwide (Siegel *et al.*, 2017). Although it is placed next to breast cancer in total incidence its predicted death rate (59%) is well above the other types of cancers (Siegel *et al.,* 2020). In general, cancer occurs by the accumulation of many genetic and epigenetic changes that are mostly linked with tumour suppressors and proto-oncogenes (Saab *et al.,* 2020). Lung cancer is broadly classified into two groups, the predominant i. non-small cell lung cancer (85% cases) and ii. small cell lung cancer (15% cases). The occurrence of non-small cell lung cancer is mostly associated with DNA mutation, followed by initiation of molecular level changes that drive the lung cancer (Herbst 2008). The most frequently mutated genes that drives non-small cell lung cancer are EGFR (10–30%), KRAS (15–30%), FGFR1 (20%), PIK3CA (2–5%), HER2 (2–5%), ALK (3%) (Levy *et al.,* 2012). In case of small cell lung cancer, mutations in two tumour suppressor genes, namely p53 and RB, are observed in the majority of cases (George *et al.,* 2015). The habit of smoking trigger the incidence of lung cancer occurrence in an individual to a level of 23 times higher than the non-smoking person (Office of the Surgeon General (US); Office on Smoking and Health (US), 2004) which are adopted by genetic changes.

Non-coding RNAs like miRNA, transfer RNA, Circular RNAs, snoRNAs, PIWI-Interacting RNAs, and YRNA that modulates many genes linked with lung cancer are identified. Among them miRNAs are extensively studied in correlation with lung cancer due to its potential biomarker ability (Braicu *et al.,* 2019). miRNA are small non-coding RNA with the size of approximately 22 nt in range that are expressed in tissue specific manner and are widely involved in the regulation of cellular metabolism, development, proliferation, and apoptosis (Zamore & Haley, 2005; Ji *et al.,* 2020). As of now many miRNAs are reported in association with lung cancer such as miR-25-3p, miR-21-5p, miR-34a-5p, miR-138-5p that regulate multiple oncogenic or tumour suppressor genes (Iqbal *et al.,* 2019). Among many miRNAs reported miR320a is critical because it is related to cisplatin resistance, which possess clinical value (Lu *et al.,* 2020). Still the role of miR320a in lung cancer is not revealed due to its controversial report of tumour suppression (Lv *et al.,* 2017) and cancer risk promotion ability (Fortunato *et al.,* 2019; Lu *et al.,* 2020). In other cancer types like breast cancer, colorectal cancer, and in hepatocellular carcinoma the miR320a expression level is downregulated and it was reported that it acts as a tumour suppressor (Xie *et al.,* 2017).

Programmed death ligand 1 (PDL1) is a 33-kDa size transmembrane protein that is usually expressed on the surface of macrophages, dendritic cells, and in a portion of activated B cells, T cells, and present in epithelial cells (Sharpe *et al.,* 2007). Additionally, PDL1 is expressed by different type of tumour cells that helps them to escape from anti-tumour responses (Ohaegbulam *et al.,* 2015). PDL1 that binds with a PD-1 receptors that are expressed predominantly on the activated cytotoxic T cells and thereby it suppress the immune system (Parra *et al.,* 2018; Shimoji *et al.,* 2016). The elevated expression of cell surface PDL1 is observed in many different cancer cells, which include non-small cell lung cancer types and it is believed that its expression helps to escape from the immune response (Shimoji *et al.,* 2016). In a recent study it was reported that miR320 modulate PDL1 level in malignant pleural mesothelioma (Costa *et al.,* 2020) but more studies are needed to conclude its role in lung cancer. The present study aims to identify the link between miR320 and PDL1 in different pathological stages of lung cancer.

MATERIALS AND METHODS

Experimental animals

One month old female A/J strain of mice (n=30) was purchased from the Jackson Laboratory, China. The mice acclimatized in the laboratory environment for 2 weeks, provided with food and water ad-libitum. Before carrying out the experiments, all the animals handling procedure and experimental protocol were approved by an animal ethical committee of Tongji University (approval No. 2017-TUSM1402216). For inducing lung cancer by means of chemical carcinogen we used Diethylnitrosamine DEN (N0258, Sigma-Aldrich), which were injected into an intraperitoneal cavity in a dose range of 25 μg/g of body weight. Following injection, one group of mice (n=10) was sacrificed after 6 months of interval and another group of mice (n=10) after 9 months for accessing the initial and advanced level of cancer development. The mice were sacrificed using cervical dislocation method following ether anaesthesia and the lung tissues were carefully dissected out. The tissues were cut into small pieces and fixed with 10% formaldehyde solution, processed with isopropyl alcohol, xylene, and finally embedded in paraffin for histopathological analysis.

Designing Locked Nucleic Acid (LNA) oligonucleotide for blocking miR320a

LNA-anti-miR320a oligonucleotide sequence of 16-mer along with modified nucleotides (5'-$u_s c_s$gcccucucaaccagcu$_s u_s u_s u_s$-Chol-3') were chemically synthesized from Axolabs. Lower case alphabet denotes 2'-O-Methyl-modified oligonucleotides, 's' stands for phosphonothioate linkage and 'Chol' specifies linked cholesterol. The obtained LNA-anti-miR320a is resuspended with 1X PBS to a final concentration of 20 mg/kg and injected through a tail vein. The injection was started following one week after Diethylnitrosamine injection and in each week four repetitive injections of LNA-anti-miR320a were given. After one week interval, LNA-anti-miR320a injection was continued again for three more weeks. For advanced stage lung cancer developing mice, similar set of LNA-anti-miR320a injections was resumed following two months of initial injection of LNA-anti-miR320a.

Immunohistochemistry

Formalin perfused, processed, and paraffin embedded tissue were subjected to sectioning using microtome (5 μm size). The sections in the glass slide following dewaxing procedure were subjected to antigen retrieval using citrate buffer solution (10 mM citric acid, pH=6.0, 95ºC) for 20 mins. The slides were washed with 1X PBST buffer and subjected to endogenous peroxidase blocking step using 3% H_2O_2 for 8–10 mins. After another wash, the non-specific sites were blocked using 5% BSA solution in 1X PBS for 1 hour at room temperature. Following that primary antibody, anti-PDL1 antibody (Abcam, ab213524) was added and incubated overnight at 4ºC. The slides were gently washed three times with a 1X PBST solution and counter react with the HRP conjugated secondary antibody at room temperature for 2 hours. After final washing, the signals in the sections were developed using the substrate 3,3-diaminobenzidine tetrahydrochloride (DAB) and final nucleus stained with haematoxylin. The slides were subjected to mounting using DPX solution and visualized under the microscope.

In-situ Hybridization

The *in-situ* hybridization experiments were performed as described earlier (Gualeni *et al.,* 2015). Initially, the sections (5 μm) in the slide were deparaffinized and treated with Proteinase K (1:200 dilution) for 20 mins. Following washing and dehydration with alcohol the slides were air-dried. The samples were hybridized using the probe against miR320a (Exiqon, double-DIG labelled LNA probes, 5'-TCGCCCTCTCAACCCAGCTTTT-3') for 2 hours at 30ºC and for control purpose scrambled miRNA probe was used. The slides were then washed with SSC buffer (saline-sodium citrate) followed by distilled water and finally with 1X reaction buffer. For imaging, the signals were developed using DAB kit and finally visualized under the microscope.

Western blot

Following the dissection of control and tumour lung tissue, it was washed with ice cold 1X PBS solution. The tissues were immediately transferred to pre-cooled mortar and pestle and ground along with the lysis buffer in the presence of protease inhibitors (Sigma-Aldrich, P8340). The final protein concentration in the protein lysate was analysed and measured using Bradford method. The SDS-PAGE wells were loaded with 60 μg of protein samples and allowed to run until it reached the dye front. The separated protein samples in the gel were transferred to PVDF membrane using semi-dry method. The efficient transfer of protein in the PVDF membrane was checked using Ponceau staining. After washing, the membrane was subjected to blocking step by being immersed in dissolved 5% milk powder in 1X TBST for 2 hours. As a next step, the membrane was transferred to a diluted primary antibody, anti-PDL1 antibody (Abcam, ab213524; 1:500 dilution) and incubated overnight at 4ºC. Following washing step, the membrane was incubated with HRP conjugated suitable secondary antibody and incubated for 2 hours in room temperature. The non-specific binding of antibody was washed away and finally developed using a DAB kit to obtain the signals. Western blotting experiments were repeated for three individual times to obtain concurrent results.

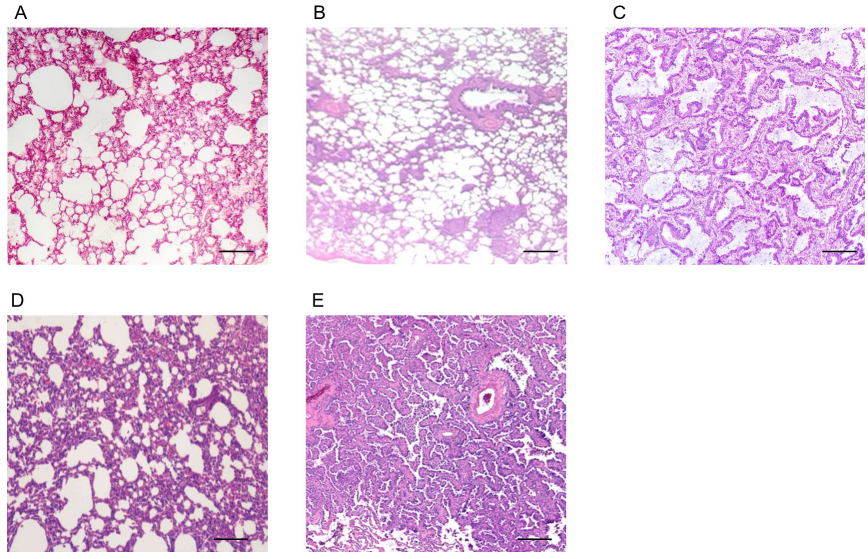

Figure 1. Histology image of lung cancer progression.
(**A**) Control lung tissue with a large gap between the alveoli cells and uniformly patterned. (**B**) Initial stage of lung cancer with neoplasm appears in between the alveoli structures. (**C**) More proliferative cells and complete cellular architecture changes observed in advanced stage lung cancer. (**D**) miRNA320a blocked initial stage lung cancer tissue shows more complex pathological changes which include tissue harness and more mitotic cells. (**E**) miRNA320a blocked advanced stage lung cancer tissue shows densely populated proliferated cells with loss in tissue elasticity. Scale bar – 100 µm

RESULTS

Chemical carcinogen mediated induction of initial and advanced lung cancer

The chemical carcinogen, Diethylnitrosamine was used in a high dose range of 25 µg/g of body weight to induce lung cancer as detailed in the materials and methods section. The single dose of Diethylnitrosamine (25 µg/g) favours the formation of initial lung cancer following 6 months of incubation time. Similarly, increasing the incubation time for up to 9 months triggers the pathological conditions similar to advanced stage lung cancer. The initial and advanced stage development of lung cancer is confirmed based on the histological examination, which shows increased pathological complexity as the incubation period prolonged (Fig. 1A–C). The control lung tissue is well organized and appears as a sponge structure with a tiny balloon like alveoli structure (Fig. 1A). However, after 6 months of Diethylnitrosamine injection, neoblast like cellular proliferations were observed and it disturbed the normal alveoli structure (Fig. 1B). Similarly, the mice injected with Diethylnitrosamine following 9 months formed a more proliferative structure that completely affected the sac like structure (Fig. 1C).

miR320a blocking elevate cancer progression

The miRNA320a were specifically blocked using complementary binding with their guide strand as described

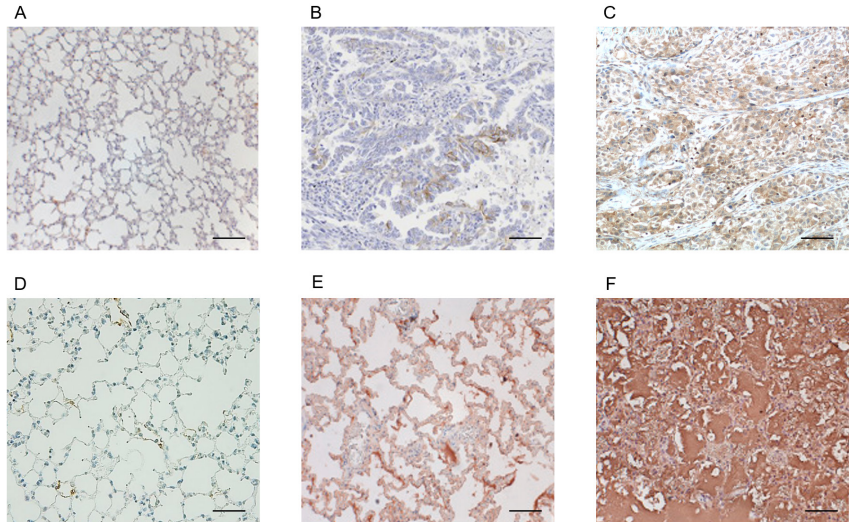

Figure 2. Immunohistochemistry image of PDL1 as lung cancer progress.
(**A**) PDL1 shows only limited positive cells for control lung tissue. (**B**) PDL1 shows marginal upregulation and observed in neoplast cells of initial lung cancer. (**C**) PDL1 is highly upregulated, and its signal is distributed all over the tissue surface of advanced stage lung cancer. (**D**) Control lung tissue shows limited signals for PDL1 in miRNA320a blocked tissue. (**E**) Overexpression of PDL1 observed in miRNA320a blocked initial stage lung cancer tissue. (**F**) Highly elevated expression of PDL1 was observed along with tissue hardness in miRNA320a blocked advanced stage lung cancer tissue. Scale bar – 100 µm

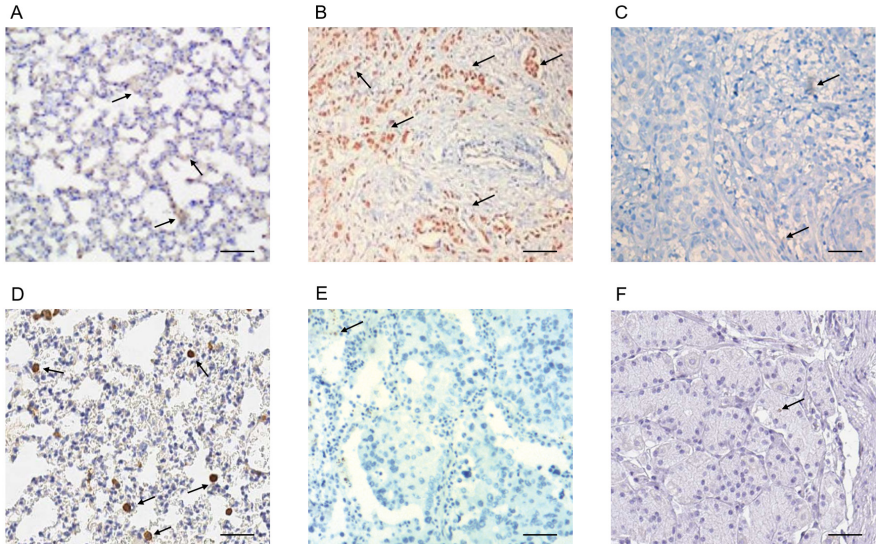

Figure 3. *In-situ* Hybridization using miRNA320a against lung cancer.
(**A**) Control lung tissue shows minimal signal for miRNA320a. (**B**) initial lung cancer tissue shows overexpressed signals for miRNA320a. (**C**) advanced lung cancer tissue shows under expressed signals for miRNA320a. (**D**) miRNA320a signal in control lung tissue following blocking of miRNA320a. (**E**) very less signal for miRNA320a but shows more histopathological complication in miRNA320a blocked initial lung cancer tissue. (**F**) miRNA320a is highly restricted in advanced stage miRNA320a blocked advanced stage lung cancer. Arrow represents signal for miRNA320a. Scale bar – 100 µm

in the materials and methods section. Following blocking of miRNA320a using LNA-anti-miR320a oligonucleotide (5'-$u_s c_s$gcccucucaacccagc$u_s u_s u_s u_s$-Chol-3'), the complexity of lung cancer was increased both in initial and advanced stage of lung cancer (Fig. 1D & E). In the initial stages of lung cancer, in addition to neoblast like appearance, the tissue seemed to harden and loose its elastic nature (Fig. 1D). In advanced stage of lung cancer, higher mitotic activity, small cells with relatively enlarged nucleus and tissue necrosis were observed (Fig. 1E).

PDL1 is highly expressed in miRNA320a blocked lung cancer tissue

As a next step, the expression of PDL1 is evaluated in initial and advanced stage lung cancer tissue as well as in miRNA320a blocked lung cancer tissue (Fig. 2A–F). In the control tissue, PDL1 expression was observed in very limited number of cells (Fig. 2A) and its expression was slightly elevated in the initial stage lung cancer tissue (Fig. 2B). The brown colour signals corresponding to PDL1 expression were observed only in neoplast cells of initial lung cancer. The expression of PDL1 is highly elevated in advanced stage of lung cancer and it is expressed throughout the tissue layers (Fig. 2C). In miRNA320a blocked control lung tissue, PDL1 expression is observed at a marginal level (Fig. 2D). But in the initial stage of lung cancer tissue, PDL1 is overexpressed to many levels along with pathological complications (Fig. 2E). Also, the brown colour signals which represent PDL1 expression is observed throughout the tissue layer. Similarly, in miRNA320a blocked advanced lung cancer tissue, along with higher proliferative cells and tissue hardiness, the PDL1 expression is multiplied with high intensity of brown coloured signal (Fig. 2F).

miRNA320a act as a tumour suppressor

The expression of miRNA32a is evaluated in lung cancer and miRNA32a blocked lung cancer tissues (Fig. 3A–F). In the control lung tissue, the miRNA320a shows limited expression (Fig. 3A) but in the initial stage of lung cancer tissue the signal for miRNA320a is overexpressed to our surprises (Fig. 3B). The brown coloured signal representing miRNA320a is highly disbursed equally throughout the tissue layer (Fig. 3B), which represents its initial tumour suppressive role. As expected, the miRNA320a expression is downregulated in advanced stages of lung cancer, which shows very limited signal (Fig. 3C). In miRNA320a blocked tissue, very weak signals only observed in control tissue (Fig. 3D) also the signals for initial and advanced stage lung cancer are highly restricted with likely less detectable signals, but it shows pathological complexity (Fig. 3E and F).

PDL1 expression analysed using Western Blotting

For analytical evaluation of increasing fold of PDL1 expression in control *vs* lung cancer tissue, Western blotting experiments were carried out and it is shown in Fig. 4. In the control tissue, PDL1 is expressed on a limited level, but is progressively overexpressed in initial and advanced lung cancer to a fold of 2.2 and 5.6-fold, respectively, higher than the control tissue (Fig. 4). Similarly, in miRNA320a blocked tissue, the PDL1 is overexpressed when compared with its respective non-blocked controls. In miRNA320a blocked control tissue PDL1 is 1.6 times overexpressed than non-blocked controls tissue. Similarly, in the initial and advanced stage of lung cancer of miRNA320a blocked tissue, PDL1 is 1.6 times and 1.9

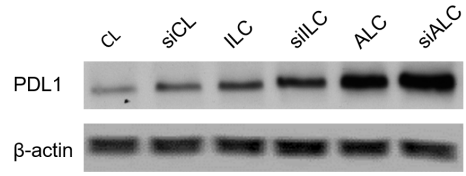

Figure 4. Western blotting against PDL1 protein.
Top row of bands represents PDL1 expression in control (CL), miRNA320a blocked control (siCL), initial lung cancer (ILC), Initial lung cancer with miRNA320a blocked (siILC), advanced lung cancer (ALC), Advanced lung cancer with miRNA320a blocked (siALC). For loading control β-actin was used.

times more highly expressed than its respective initial and advanced miRNA320a non-blocked tissue.

DISCUSSION

From our investigation it was shown that the single dose of Diethylnitrosamine (25 μg/g) is proven to induce initial and advanced stage lung cancer following 6- and 9-months incubation. Chemical carcinogen, Diethylnitrosamine is able to induce mutation and the incubation period is the key in determining the progression of lung cancer. In addition, the A/J strain of mice that were used in the present experiment is more susceptible to chemical carcinogen (Hecht *et al*., 1994) therefore we used only half of the dose of Diethylnitrosamine (25 μg/g), at the same time avoiding the very minimal dose (Mervai *et al*., 2018). The activity of Diethylnitrosamine in the dose of 25 μg/g is further evident with the histopathological complication as the incubation time is prolonged.

As the stages of lung cancer progress, more proliferative cells form a neoplast like structure. Especially in the advanced stage of lung cancer, the neoplast forms solid structure with more necrotic cells and without its elastic ability (Popper 2015; Popper 2016). In our investigation, we observed that the upregulation of PDL1 expression is highly elevated in advanced stage of lung cancer when compared with initial lung cancer. When connecting our experimental data with the previous study data that the immunotherapies against PDL1 works well at an advanced stage of lung cancer (Yu *et al*., 2016) revealed that higher expression of PDL1 happens only in the advanced stage of lung cancer. The immunohistochemical assessment of PDL1 expression is unclear, misleading, and it shows no significant value for the prognosis (Tang *et al*., 2015). Therefore, understanding its expression should be studied with their closely regulating molecules that govern PDL1 expression.

The link between PDL1 and miRNA320 was revealed from our study and it shows that blocking miRNA320 expression triggers lung cancer both in initial and advanced stages of lung cancer by regulating PDL1 overexpression. PDL1 testing is a puzzle and controversy related to sample collection timing, type of cells to be accessed, different staining methods (Teixidó *et al*., 2018) and here we point out that the expression of PDL1 is multi fold increased in advanced stage of lung cancer. Additionally, the correlation between miRNA320 and PDL1 is revealed by blocking assay, which shows that miRNA320 act as a tumour suppressor and it regulates PDL1, because miRNA320 overexpression in initial lung cancer underregulate the PDL1 expression and its suppression favours PDL1 overexpression. A similar finding has also recently emerged that in the case of malignant mesothelioma miRNA320a expression downregulates PDL1 (Costa *et al*.. 2020). In some studies, the higher expression of miRNA320a is understood misleadingly as it promotes lung cancer (Fortunato et al. 2019) but it is clearly observed in correlation with PDL1 that it suppresses the lung cancer.

In conclusion, we successfully induced initial and advanced stage lung cancer using the chemical Diethylnitrosamine (25 μg/g). The miRNA320a negatively regulate PDL1 which are confirmed with the overexpression of PDL1 in miRNA320a blocked tissue.

Declarations

Conflict of interest. We declare that the authors have no conflict of interest among themselves.

Authors Contribution. Liyun Xu, contribute to designing the study and fund mobilization. Zhiyi Dong, contribute to performing major experiments. Weiqing Gu, contribute to doing experiments, Liyun Xu, perform statistical analysis and critical review of the manuscript. Zhiyi Dong, provide support in doing minor experiments.

Ethics approval and consent to participate. Animal studies are approved by animal ethical committee of Tongji University (approval No. 2017-TUSM1402216).

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