Review article

P62: An emerging oncetarget for osteolytic metastasis

Jing Zhang a, Zuozhang Yang a, Jian Dong a,b,*

a Bone and Soft Tissue Tumors Research Center of Yunnan Province, Department of Orthopaedics, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan Province, Kunming, Yunnan 650118, PR China
b Stem Cell Therapy Technical of Clinical Transformation and Basic Research Key Laboratory of Yunnan Province, Kunming, Yunnan 650118, PR China

A R T I C L E   I N F O

Article history:
Received 17 November 2015
Received in revised form 18 January 2016
Accepted 30 January 2016
Available online 1 February 2016

Keywords:
P62/SQSTM1
Bone metastasis
Cancer
Osteoclasts
Autophagy

A B S T R A C T

Bone metastasis occurs in the majority of late-stage tumors with poor prognosis. It is mainly classified as osteoblastic metastasis and osteolytic metastasis. The pathogenesis of osteolytic metastasis is a “vicious cycle” between tumor cells and bone cells (primarily the osteoclasts), which is mediated by secretory factors. The P62 adapter protein is a versatile multitasker between tumor cells and bone cells. The overexpression of P62 has been detected among a variety of tumors, playing positive roles in both tumorigenesis and metastasis. Moreover, P62 is an important modulator of the osteoclastogenesis pathway. Therefore, the ability of P62 to modulate tumors and osteoclasts suggests that it may be a feasible oncetarget for bone metastasis, especially for osteolytic metastasis. Recent research has shown that a P62 DNA vaccine triggered effective anti-tumor, anti-metastatic and anti-osteoporotic activities. Growing lines of evidence point to P62 as an emerging oncetarget for osteolytic metastasis. In this review, we outline the different roles of P62 in tumor cells and osteoclasts, focusing on the P62-related signaling pathways in key steps of osteolytic metastasis, including tumorigenesis, metastasis, and osteoclastogenesis. Finally, we discuss the newest observations on P62 as an oncetarget for osteolytic metastasis treatment.

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* Corresponding author at: Bone and Soft Tissue Tumors Research Center of Yunnan Province, Department of Orthopaedics, The Third Affiliated Hospital of Kunming Medical University, Tumor Hospital of Yunnan Province, Kunming, Yunnan 650118, PR China.
E-mail address: njmlimit@sina.com (J. Dong).

http://dx.doi.org/10.1016/j.jbo.2016.01.003
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lesions in bone are significantly associated with bone pain, hypercalcemia or hypocalcemia, pathological fracture and spinal cord compression, which mostly correlated to low 5-years survival-rate less than 30% [2]. Relative to osteoblastic metastasis, osteolytic metastasis is more difficult to treat, making the need to study its pathogenesis. Recent advances in the understanding of osteolytic metastasis revealed that it is associated with characteristic modulations of the bone microenvironment and cross-talk between the tumor cells and bone cells (primarily the osteoclasts). Tumor cells condition the “metastatic niche” through the secretion of soluble factors that stimulate bone resorption by the osteoclasts. Osteostastic bone resorption results in the release and activation of growth factors in the bone microenvironment that further stimulate tumor growth, leading to a “vicious cycle” [3]. It is involved with numerous signaling factors, but the crucial molecules are still uncertain [1].

Since its initial discovery as an atypical protein kinase C (PKC)-interacting protein, P62 (also known as sequestosome-1, SQSTM-1 or A170) has emerged as a crucial molecule in the regulation of cell growth, survival and proliferation [4]. The human P62 protein has 440 amino acid residues and contains different types of protein–protein interaction domains. The multi-functional domains of the P62 adapter protein are consistent with its role as a versatile multitasker in signal transduction in tumors [5], (Fig. 1). P62 accumulation promotes tumorigenesis [6]. It is found to be either not expressed or found at low levels of expression in normal tissues, but is over-expressed among various types of tumors and, for the most part, is correlated with tumor migration, invasion or metastasis [7–24]. Thus, the P62 gene is generally acknowledged to be an oncogene. In addition, many studies on Paget’s disease of bone (PDB), which is a skeletal disorder characterized by osteolytic lesions and overactive osteoclasts, have identified P62 as an important modulator of the osteoclastogenesis pathway [25–27]. The dysregulated expression of the P62 protein promotes osteoclastogenesis, bone resorption and osteolytic lesions. Therefore, P62 has long been thought of as a promising molecular target in PDB and other bone metabolic diseases [28].

Many in vitro and in vivo studies employing knockdown have shown that P62 can inhibit tumor formation, proliferation and/or progression [29–31]. Recent research on intramuscularly or intravenously administered P62 DNA vaccines showed that they induced anti-P62 antibodies and exhibited strong anti-tumor and anti-metastatic activities in transplantable mouse tumors [32] and canine spontaneous mammary neoplasm models [33]. The latest research reported the unexpected finding that intramuscular delivery of P62 DNA vaccines exerts a powerful anti-osteoporotic activity in a mouse model of inflammatory bone loss [34]. These studies promoted P62 as an oncotarget for bone metastasis, especially for osteolytic metastasis.

In this review, we outline the different role of P62 in tumor cell and osteoclast, focusing on the P62-related signal pathway in key steps of osteolytic metastasis, including tumorigenesis, metastasis and osteoclastogenesis. Finally, we discuss the newest observations about P62 as an oncotarget for osteolytic metastasis treatment.
2. The role of P62 in tumors

2.1. P62 accumulation promotes tumorigenesis

P62 is over-expressed among almost all differently primary tumors, including prostate [7–9], breast [10–13], lung [14], kidney [15], head and neck [16], esophageal [17], gastric [18], liver [19], colon [20], and ovarian [21] tumors, and myelomas [22], melano- mas [23], and glioblastomas [24]. This implies that P62 may play an extensive role in tumorigenesis. Using immunohistochemical staining and an enzyme-linked immunosorbent assay, P62 over-expression was shown to be localized to the tumor cell cytoplasm and nucleus [12,22,24], which suggested that P62 may shuttle in transcription and translation signaling of tumor cells.

First, P62 promotes tumorigenesis through the autophagy pathway. Autophagy is a homeostatic process that occurs in all eukaryotic cells and involves the sequestration of cytoplasmic components (proteins and organelles) in double-membraned autophagosomes [35]. P62 has been shown to be both an autophagy substrate and an autophagy adapter protein that acts as a link between ubiquitination and autophagy. Defects in autophagy promote a failure of protein and organelle quality control in cells, which leads to P62 accumulation, resulting in perturbation of gene transcription and translation signaling of tumor cells.

Second, P62 is required for Ras-induced tumorigenesis. The Ras proto-oncogene has been found to be mutated in at least 25% of human tumors and is a potent activator of NF-κB, which is important for cell survival and tumor transformation [40]. P62 is an important NF-κB mediator of Ras-induced tumorigenesis [41,42]. In a model of Ras-induced lung adenocarcinoma, in vitro and in vivo, P62 was induced by Ras to trigger IκB kinase (IκK) through the polyubiquitination of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). Then, activation of NF-κB increased inflammation and tumorigenesis. Deficiency of P62 also accounted for enhanced cell death and reduced the tumorigenicity of Ras [41]. Another recent study found that P62 plays an important role in the development of pancreatic ductal adenocarcinomas through a feedforward loop, whereby Ras activates NF-κB; NF-κB then transcriptionally induces P62 [42]. Therefore, P62 is a positive mediator in Ras-induced tumorigenesis by activation of NF-κB.

Finally, P62 is a new mediator of oxidative stress signaling in tumorigenesis. Oxidative stress is common in tumor microenvironment. Oxidative stress produces additional reactive oxygen species (ROS), which increase tumorigenesis through the induction of genome instability [43]. As an emerging regulator at the interface of oxidative stress signaling and tumorigenesis, P62 was shown to interact with Kelch-like ECH-associated protein 1 (KEAP1) at the transcription factor NF-E2-related factor 2 (NRF2) binding site, thereby competing with KEAP1 binding and thus promoting NRF2 release from KEAP1 [44]. Finally NRF2 activation induced many antioxidant gene expressions, which promoted cell survival and tumorigenesis [45]. Additionally, animal models with genetically-impaired autophagy initiation also reveal P62 accumulation, prolonged NRF2 activation, which led to tumor cell survival and tumorigenesis [46].

Recently, we also reviewed complex correlation between autophagy and cancer metastasis [47]. Autophagy may serve as a double-edged sword depending on the contextual demands placed on tumor cells throughout the metastatic process [48]. Latest research implied autophagy may play a role in cancer metastasis via promoting metastatic colonization [49]. P53 status may determine the role of autophagy in tumor development [50]. When p53 is functional, autophagy is required for tumor progression, but that autophagy might function as part of the tumor-suppression machinery when the function of p53 is lost [51].

P62 is over-expressed in primary tumor tissues, but there is little research on P62 expression in metastatic tumor tissues. The results appear to be contradictory. Kim evaluated autophagy activity in pulmonary metastasis of colorectal tumors and compared recurrence and non-recurrence groups. The results showed enhanced autophagy activity and decreased P62 expression in the recurrence group [52]. Another study showed there was no difference in P62 expression according to histologic subtypes in metastatic tumor tissues of unknown primary tumors [53]. However, in a mouse colorectal lung metastatic model, P62 levels were increased because autophagy activity was inhibited [54]. Generally speaking, in established tumors, autophagic responses constitute a means to cope with intracellular and environmental stress, thus favoring tumor metastasis [47,55]. As a substrate protein of autophagy, overactive autophagy leads to P62 degradation. Therefore, it seems that P62 expression is negatively correlated with tumor metastasis.

However, in autophagy deficient cells, P62 plays different roles in tumor progression or metastasis. Qiang demonstrated that, in autophagy deficiency cells, P62 accumulation promotes cell proliferation and migration through P62-dependent stabilization of the oncogenic transcription factor Twist1, and in a mouse model, P62 up-regulation promoted tumor growth and metastasis [56]. Another study showed that interrupting P62 with TRB3 causes P62 accumulation, which produces potent anti-tumor efficacies against tumor growth and metastasis [57]. Interestingly, recent research demonstrated that P62 and autophagy synergize to promote tumor growth and metastasis. In autophagy-deficient established murine tumors, increased P62 potentiated NF-κB signaling and the elaboration of pro-tumorigenic inflammatory cytokines to promote tumor growth and metastasis [58]. Therefore, “doubling down” on the P62 and autophagy pathway reveals an unexpected synergism [59]. It could be more effective than targeting either pathway alone to suppress tumor growth and metastasis. We then speculated P62 might play a dual role in tumor metastasis: under active autophagy status, as an autophagy receptor and substrate, P62 degradation promotes tumor metastasis, but under deficient autophagy status, P62 accumulation potentiates NF-κB signaling to promote tumor metastasis.

3. The role of P62 in osteoclastogenesis

3.1. P62 plays a positive role in osteoclastogenesis through the NF-κB activation pathway

Bone is continuously renewed through the dynamic balance between bone resorption and bone formation. Osteoclasts play a
P62 is a protein that modulates protein–protein interactions using its different types of protein interaction domains. Three of the domains; i.e., PB1, ZZ and TB, interact with the RANKL--RANK--NF-κB pathway. The PB1 domain of P62 binds PKCζ, through a PB1–PB1 interaction, the ZZ finger domain binds receptor interacting protein (RIP), and the TRAF6 binding (TB) domain binds TRAF6. These three domains are relevant in the canonical and noncanonical NF-κB activation pathways of osteoclastogenesis (Fig. 3).

P62 gene mutation was considered to be the main cause of Paget's disease of bone (PDB) [25–28], which is a skeletal disorder characterized by osteolytic lesions and overactive osteoclasts. To date, 28 distinct P62 mutations have been reported in PDB patients and the most commonly identified mutation leads to a proline to leucine substitution at residue 392 (P392L) [28,68]. Dysregulated expression of P62 protein promotes osteoclastogenesis, bone resorption and osteolytic lesions through the NF-κB activation pathway, leading to focal areas of aberrant and excessive bone turnover of PDB patients [69]. In vivo, six- to-eight-week-old P62 knockout mice showed complex signs caused by inactivation of the osteoclasts, as well as inhibition of IKK activation and NF-κB nuclear translocation [70]. Therefore, it is certain that P62 plays a positive role in osteoclastogenesis through the NF-κB activation pathway.

### 3.2. P62 may play an essential role in RANKL-induced autophagy and osteoclastogenesis

Autophagy plays a changing role in osteoclastogenesis. Recently, the roles of autophagy in the function of cartilage cells and bone cells (primarily the osteoclasts, osteoblasts, and osteocytes) were reviewed exhaustively [71,72]. Autophagy has dual roles, regulating the osteoclast precursor cell pool and subsequent osteoclast formation and activation. On the one hand, the activation of autophagy promotes osteoclast formation and function. The deletion of ATG7 and ATG5 reduced the formation and resorptive capacity of osteoclasts in conditional knockout mice [73,74]. On the other hand, the induction of autophagy may negatively regulate both osteoclast formation and activity. Treatment with rapamycin (which induces induction of autophagy may negatively regulate both osteoclast formation and activity. Treatment with rapamycin (which induces autophagy and osteoclastogenesis) was changed according to autophagy activity [77].

A recent study investigated the correlation of P62-autophagy–osteoclastogenesis. Using RT-PCR and Western-blots immunofluorescence analyses, the data showed that the expression of P62 was significantly altered during RANKL-induced osteoclast differentiation. Importantly, the knockdown of P62 obviously attenuated RANKL-induced expression of autophagy- and osteoclastogenesis-related genes. These indicated that P62 may play essential roles in RANKL-induced autophagy and osteoclastogenesis [78].

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**Fig. 3. Osteoclastogenesis induced by adapter P62 in NF-κB activation pathway.** RANKL activates both canonical and noncanonical pathways of NF-κB in osteoclastic like cells. In the canonical pathway, RANK and IL-1 binding to RANK leads quickly TRAF-6 binding to TB domain of P62. Deubiquitinating enzyme CYLD targets TRAF6 via its interaction with the UBA domain of P62. P62 activated aPKC, which induce activation of IKK and IκB, IκBα consequently undergoes rapid degradation by proteasome, resulting in release of p65 and p50 and their translocation to nuclei where they prevent apoptosis of osteoclast precursors, thus allowing them to continue differentiating. In the noncanonical pathway, RANK binding to RANK interacts with TRAF3, activation of the NF-κB inducing kinase (NIK) and IκKα, lead to the phosphorylation of p100 and the processing of p100→p52.
3.3. P62 plays a novel role between oxidative stress and osteoclastogenesis

In oxidative stress, reactive oxygen species (ROS) are crucial in bone homeostasis through the stimulation of osteoclasts, the inhibition of osteoblasts, and the induction of osteocyte apoptosis [79]. RANKL induces the production of long-lived ROS that are crucial for the late stages of osteoclastogenesis and regulation of bone resorption [80]. As previously mentioned, P62 was a novel mediator in KEAP1/NRF2 signaling of oxidative stress [44]. A recent study showed that the S349T mutation of P62 linked KEAP1/NRF2 signaling to PDB. The S349T mutant P62 showed reducing ability to activate NRF2 signaling in differentiating osteoclast-like cells [81]. Therefore, NRF2 deficiency promoted RANKL-induced osteoclast differentiation [82]. This may be a new mechanism for the role of P62 in PDB [68]. On the other hand, the correlation between oxidative stress and autophagy has long been known [83]. As a key role in oxidative stress and autophagy crosstalk, the phosphorylation of P62 activates the NRF2-KEAP1 pathway during selective autophagy [84]. Interestingly, the P62 NRF2-autophagy regulation appears to rely on dual feedback loops. First, a positive feedback loop is the regulation of P62 expression by NRF2, which in turn regulates NRF2 stabilization and degradation [85]. Therefore, P62 regulates its own transcription by controlling NRF2 activation. Second, starvation has been reported to induce ROS formation, which in turn triggers autophagy [86]. It creates a negative feedback loop whereby, under the conditions of starvation, ROS formation stimulates autophagy, which will result in the degradation of P62 and the attenuation of NRF2 activation [45]. In short, P62-NRF2-autophagy feedback loops may play a novel role in oxidative stress and osteoclastogenesis.

4. P62 as an oncotarget for treatment

4.1. Knockdown of P62 for tumor and bone metastasis

As previously mentioned, the multi-functional domains of the P62 adapter protein determine its role as a versatile multitasker in signal transduction [5]. P62 plays different roles in tumorigenesis, osteoclastogenesis, autophagy and oxidative stress. In theory, knockdown of P62 expression would lead to intracellular reactions. However, the existing research showed knockdown of P62 expression was an attractive target for tumor and bone disease. The earlier studies found that some medicines, such as chloroquine [87] and calpain inhibitor [88], induced anti-tumor activity through P62 clearance. Later studies showed RNAi silencing of P62 significantly inhibited tumor growth both in vitro and in xenograft tumors model [29–31]. A puzzling aspect of the anti-tumor mechanism is interaction between P62 and autophagy. Down-regulation of P62 showed anti-tumor activity, but it also activated autophagy, which promoted tumor growth conversely. Nihira studied P62 inhibition on the regulation of autophagy and cell survival in P62-positive carcinoma cells. The results showed P62-silencing dramatically suppressed cell proliferation and cell survival in P62-positive carcinoma cells. However, this autophagy is deficient, which was manifested as formation of mis-regulated autophagosomes with multilayer membranes and an autophagic cell death [29]. Islam showed similar results in a K-ras341 mouse model. Using an osmotically active polysorbitol-mediated transporter (PSMT) to downregulate P62 by an RNAi strategy, autophagy was over-activated and remarkably reduced the size and number of tumors by suppressing proliferative cell nuclear antigen, CD 31, and VEGF levels [30]. In short, knockdown of P62 was a promising strategy for tumor targeted treatment.

The ability of P62 to modulate tumors and osteoclasts suggests that it may be a feasible oncotarget for bone metastasis, especially for osteolytic metastasis. First, P62 is required for the cell survival of apoptosis-resistant bone metastatic prostate tumor (PCa) cells. In bone metastatic lines that escape the bone marrow stromal cell paracrine factors (BMSC-PF), induced apoptosis can upregulate cytoprotective autophagy. In these PCa cell lines, the BMSC-PF signaling upregulated the P62 mRNA and protein by IL-1β [89], the siRNA knockdown of P62 was cytotoxic and the immunostaining showed a peri-nuclear clustering of the autolysosomes. Therefore, P62 could be exploited to target and kill these apoptosis-resistant PCa cells in the bone [9]. Secondly, P62 is also an attractive therapeutic target for multiple myeloma (MM) that is characterized by osteolytic destruction. Knocking-down P62 in patient-derived stromal cells significantly decreased the PKCζ, VCAM-1, and IL-6 levels as well as decreasing the stromal cell support of MM cell growth. Similarly, marrow stromal cells from P62−/− mice produced much lower levels of IL-6, TNF-α, RANKL, and supported MM cell growth and osteoclast formation to a much lower extent than in normal cells [22]. The latest study reveals that P62 aggregation is a faithful marker of defective proteostasis, defining a novel prognostic and therapeutic framework for MM [90]. An exciting recent study showed that osteolytic metastasis is hampered by impinging on the interplay among autophagy, anoikis and ossifications. Osteolytic metastasis was largely prevented because of an autophagy failure marked by P62 without Beclin-1 [91]. However, an accurate molecular mechanism is still unclear.

4.2. P62 vaccine for tumor and bone disease treatment

The abundant studies on P62, tumors and PDB raises the possibility of a P62 vaccine for tumor and bone disease treatment. In 2013, Franco first performed a preclinical study of a novel DNA vaccine encoding P62 [32]. Surprisingly, the P62 vaccine showed broad-spectrum anti-tumor and anti-metastatic activity. Intramuscularly or intravenously administered P62 DNA vaccine induced anti-P62 antibodies and exhibited strong antitumor activity in four models of allogeneic mouse tumors; i.e., B16 melanoma, Lewis lung carcinoma, S37 sarcoma, and Ca755 breast carcinoma. A P62 DNA vaccine also dramatically decreased the number or sizes (volumes) of lung or lymphatic metastases. Then, Vladimir analyzed the feasibility a P62 DNA vaccine as a tumor immunotherapy target [92]. P62 protein fits the following antigen criteria: (i) immunogenic; (ii) essential for tumors cells (to avoid its loss through immune-editing), but dispensable for normal tissues to reduce the risk of toxicity; and (iii) overexpressed in tumors compared to the normal tissues. They subsequently demonstrated that P62 DNA plasmids, when administered in a preoperative setting, decreased and/or stabilized growth of advanced lesions in canine mammary tumors. These studies proved the P62 vaccine not only had good antitumor and anti-metastatic activity but also little toxicity [32,33]. Recently, this team reported an unexpected finding that intramuscular delivery of a P62 vaccine exerts a powerful anti-osteoporotic activity in a mouse model of inflammatory bone loss by combining bone-sparing and osteosynthetic effects. Notably, the suppression of osteoporosis by a P62 vaccine was associated with sharp down-regulation of master inflammatory cytokines, and up-regulation of endogenous P62 protein by bone-marrow stromal cells. However, the study failed to detect the osteoclast activity when the P62 vaccine was administered in vivo [34]. On the whole, the data provide a solid rational to apply a P62 DNA vaccine as a safe, new therapeutic treatment for tumor and bone loss diseases. Therefore, in an osteolytic metastasis model, which was characterized by tumor and bone loss disease, we could hypothesis that a P62 vaccine may exert a combined effect in osteolytic metastasis treatment.
5. Concluding remarks

P62 is a cellular “Swiss army knife,” with its diverse cellular functions arising from its unique functional motifs and protein–protein interaction properties. P62 may play different roles in different type cells or different states of cells. For example, in tumor cells, P62 accumulation results in perturbation of gene expression, increased genome damage and tumorigenesis [6]. However, in tumor stromal cells, P62 up-regulation is an anti-inflammatory tumor suppressor that acts through the modulation of metabolism [93]. At the same time, its multifunctional nature may contribute to its complex role in osteolytic metastasis. For example, the up-regulation of P62 can promote osteoclastogenesis and differentiation by activating RANKL–RANK-NF-κB signaling, thereby accelerating bone resorption. This implies that P62 may promote osteolytic metastasis. However, as a receptor protein of autophagy, P62 accumulated because of autophagy failure, which hampered osteolytic metastasis. This implies that P62 may be, contradictorily, a reducer of osteolytic metastasis. In another example, as previously noted, the P62-NRF2-autophagy signal feedback loop includes two contradictory types, also playing an important role in osteoclastogenesis, and appears to be similar to the P62-mTOR-autophagy feedback in fat metabolism [94]. As a whole, these apparently contradictory roles showed that P62 is a multi-domain protein that may play different roles in different types of cells and in different microenvironments. Nevertheless, the ability of P62 to modulate tumors and osteoclasts suggests that it may be a feasible oncotarget for bone metastasis, especially for osteolytic metastasis. P62 may be an attractive target for therapeutic intervention if we are able to selectively modulate the interactions of P62 with specific signaling molecules, perhaps by using different interaction modules inherent in its structure.

Molecule targeted therapy is an important developing direction for tumor treatments, and the key to its success is the identification of crucial molecular targets. The mechanism of osteolytic metastasis involves different types of cells and intricate molecular signaling. P62 is at the interface of both tumor cells and osteoclasts, at the juncture of different signaling pathways that are correlated with tumorigenesis, metastasis and bone turnover. Therefore, selective regulation of P62 expression may be a feasible strategy for bone metastasis, especially for osteolytic metastasis. To date, several animal studies using the P62 vaccine showed broad-spectrum anti-tumor, anti-metastatic and anti-osteoporotic activity. A number of human clinical trials of DNA vaccines have been performed or are ongoing for tumors and showed encouraging results [95]. Further studies should be designed to detect P62 expression in osteolytic metastasis tissues. Meanwhile, evaluating the curative effects of down-regulating P62 expression in osteolytic metastasis animal models may provide an experimental basis for new targeted treatments.

Competing interests

The authors declare that they have no competing interests.

Authors contributions

Study concept and design: Jian Dong, Jing Zhang. Figure Preparing: Zuozhang Yang, Jing Zhang. Manuscript preparation: Jing Zhang. Manuscript review: Jian Dong. All authors have read and approval the final manuscript.

Acknowledgments

This research was supported in part by grants (no. 81302343/H1624) from the National Natural Science Foundation of China, a Grant (no. 2014FB0 592014FB067) from the Joint Special Funds for the Department of Science and Technology of Yunnan Province – Kunming Medical University.

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