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Original Article

Expression of neuropeptide Y and vasoactive intestine peptide in pathological tissues of congenital pseudarthrosis of the tibia and its clinical significance

Hongyi Qu[1*], Kelai Wang[2*], Yan Zhuang[2*], Juntao Ge[1], Zuohui Zhao[1], Yue Xu[1]

[1]*Shandong Provincial Qianfoshan Hospital, Jinan, Shandong, China;* [2]*Qilu Hospital, Shandong University, Jinan, Shandong, China.* [*]*Equal contributors.*

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Abstract: Background: We aimed to determine the expression of two neuropeptides, neuropeptide Y (NPY) and vasoactive intestine peptide (VIP), in the pathological tissues of congenital pseudarthrosis of the tibia (CPT) patients for understanding the etiology and pathogenesis of CPT. Methods: Periosteum and bone specimens from 20 CPT patients were studied, in comparison to 20 normal periosteum and bone tissues as control. Immunohistochemistry staining to determine the expression of NPY and VIP was performed and the differences between in the CPT and control groups were compared. Results: NPY was expressed at blood vessel wall and periphery of vessels in periosteum and bones. NPY expression levels were higher in periosteum of CPT than that in control group. NPY was not expressed in bones of CPT and control groups. VIP was expressed both in periosteum and bone tissues. The levels of VIP expression in CPT periosteum were significantly less than those in the control group. Conclusion: VIP expression was significantly higher in osteoclasts of CPT than those of control. Abnormal expression of NPY and VIP in CPT tissues may be a causal factor of CPT.

Keywords: Neuropeptide Y (NPY), vasoactive intestine peptide (VIP), congenital pseudarthrosis of the tibia (CPT)

Introduction

Congenital pseudarthrosis of the tibia (CPT) is a rare orthopedic disorder in children with an incidence rate ranging from 1:140,000 to 1:250,000 births [1]. Despite its first description in 1891, CPT is still a disease difficult to treat and the etiology and pathogenesis of CPT are poorly understood [1]. In recent years, neuropeptides released by peptidergic neurons has attracted growing attention due to the role of neuropeptides in regulating bone metabolism [2]. Konttinen et al. reported the existence of neuropeptide receptors in healthy bone tissues including periosteal blood vessels, osteoblasts and osteoclasts [3]. Neuropeptides, by binding their receptors in the target cells, cause expansion or contraction of blood vessels to regulate blood flow, and induce differentiation of osteoblasts and osteoclasts to regulate bone metabolism. Despite the studies, the role of neuropeptide in bone metabolism of CPT patients is still little understood. The main purpose of this study is to detect the expression of two neuropeptides, neuropeptide Y (NPY) and vasoactive intestine peptide (VIP), in CPT lesions using immunohistochemistry. The contribution of neuropeptides in the etiology and pathogenesis of CPT was discussed.

Materials and methods

Preparation of specimens

Specimens of the CPT group: The specimens were the paraffin blocks of CPT tissues collected during the first surgery on 20 CPT patients who were admitted to Department of Pediatric Surgery, Qilu Hospital during the past 30 years. According to the case records and the pathologists' observation of the HE sections, the paraffin block specimens were the pathological periosteum tissues and the bone tissue around the false joints. The patients' age ranged from 6 months to 6 years, with an average of 3.0 years. Among the 20 cases, 14 were male and 6

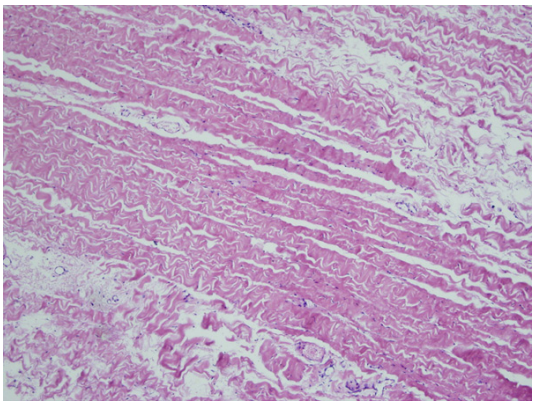

Figure 1. The fibrous layer of the periosteum of the CPT cases (100 × magnification). The fibrous layer was abnormally thickened and collagen fibers were disordered.

Table 1. Mean vessel density (MVD)

	CPT periosteum (CPT group)	Normal periosteum (control group)
MVD	40.296±1.848①	50.263±1.537①

①: There was statistically significant difference within the group ($P < 0.05$).

females. And 12 cases were from the unilateral left body side and 8 were from right side, with no bilateral case. All CPT specimens have no visible milk-coffee spots. All 20 patients had no history of trauma and were all Boyd II type, based on the X-ray images. The surgery involved pseudarthrosis-cutting, autologous bone graft, internal fixation of bone nail, external fixation with plaster. During surgical operation, abnormal thickening of the periosteum, tapering of the fractured ends, sclerization, and disappearance of marrow cavity were observed. Pathological diagnosis of the tissue removed included proliferation of fibrous connective tissue, appearance of osteotylus and broken bone fragments.

Specimens of control group: Normal periosteum and bone tissues from 20 individuals were used as the control. These include the unilateral fibular periosteum and bone tissue without fibular lesions from 14 children diagnosed with CPT, and the periosteum and bone tissue of tibia from 6 children with genu varum who underwent tibial osteotomy. The children's ages ranged from 1 to 8 years, with an average of 3 years and 7 months.

Methods

Preparation of paraffin slices: Tissue specimens were fixed, decalcified, dehydrated, cleared, embedded in paraffin. Slices (4 μm) were cut and stained using the standard hematoxylin and eosin (HE) staining method. Then the stained slices were examined by pathologists to confirm that the preparation process was appropriate and the slices were suitable for subsequent immunohistochemical staining.

NPY, VIP immunohistochemical staining: Monoclonal antibodies against NPY (Boster Inc. Wuhan, China) and VIP (Zhongshan Inc, Beijing, China) were used as the primary antibodies, each diluted 1:100. The standard Streptavidin-Biotin Complex (SABC) method was used for the staining.

Image acquisition and analysis: The image acquisition and analysis were performed by two independent pathologists in a randomized, double-blind manner. Upon finishing the immunohistochemical staining, true-color images of the tissue slices were analyzed using software (Image Pro Plus 5.0, Media Cybernetics Inc.) to quantify the expression levels of NPY and VIP in tissues. The expression levels of NPY and VIP were indicated with two semi-quantitatively indices, mean positive area (MPA) and mean optical density (MOD). In other words, for each neuropeptide, bigger MPA and MOD values indicated higher expression levels.

Upon finishing HE staining, mean vessel density (MVD) as an index of periosteal vascular density was acquired according to the procedure of Tamas et al. [4]. Briefly, five fields of the same size were randomly chosen from within the most vessel-dense region of the HE-stained tissue specimen under the bright-field optical microscope at low magnification (40 ×). Subsequently, the number of vessels within each field was counted under the microscope at high magnification (400 ×). The average number of vessels in five fields of the slice is the MVD of the slice. The average of vascular density of all slices in each group is the MVD of the group.

Statistical analysis: Statistical analyses were performed using the statistical software SPSS13.0. After calculating the mean and standard deviation of each group, the differences

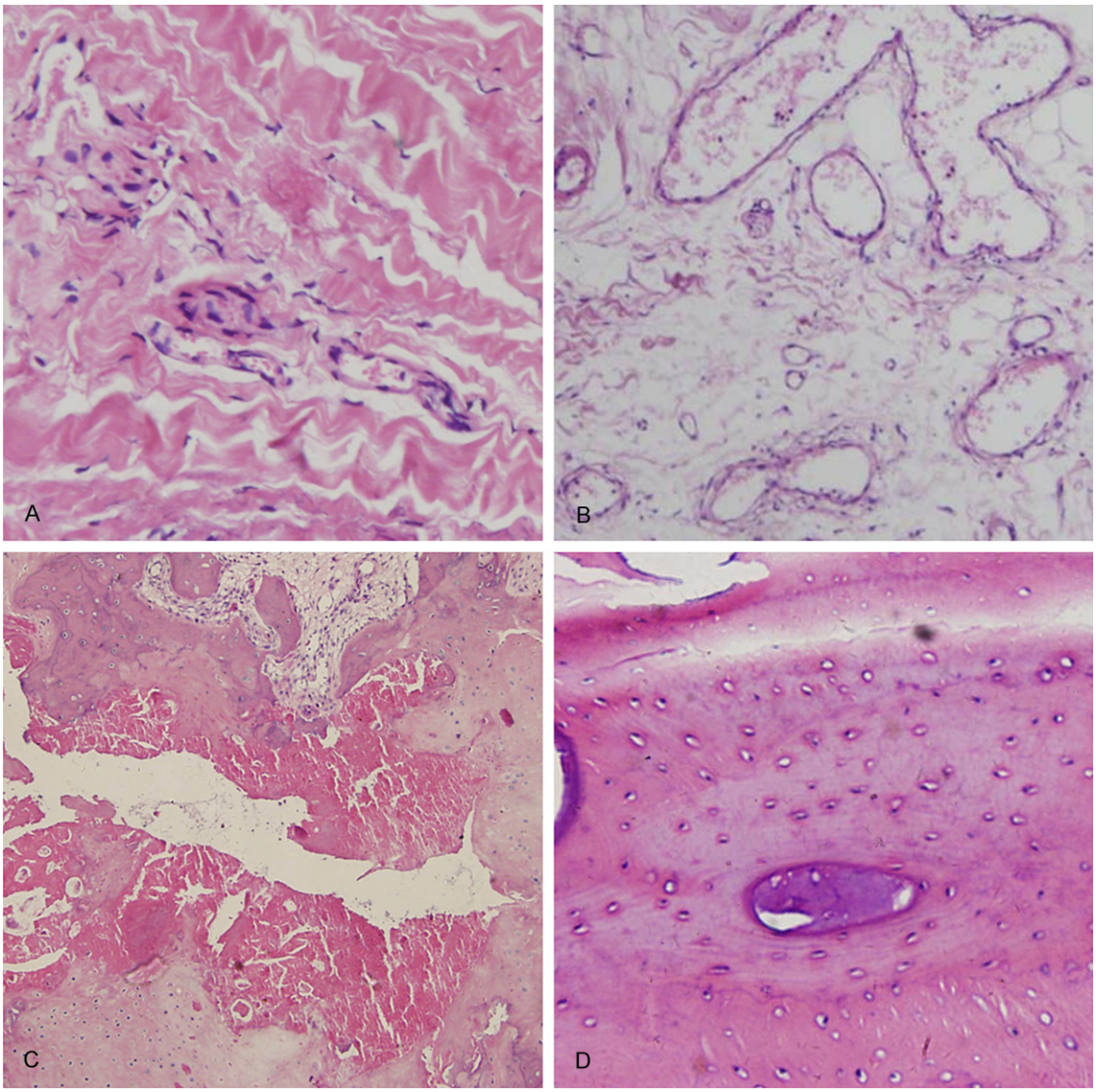

Figure 2. A. Blood vessel of CPT periosteum (400 ×). Vessel diameters were decreased and vessel stenosis was visible; B. Blood vessel of normal periosteum; C. CPT bone tissue (100 ×). Disorganized trabecula, fibrous connective tissue and bone necrosis ingredients were visible in the CPT bone tissue; D. Normal bone tissue (200 ×).

between the CPT and control groups were analyzed using Student's t test, with *P* values smaller than 0.05 indicating statistically significant differences.

Results

Hematoxylin and eosin (HE) staining of the specimens

The medium-outer fiber layers of normal periosteum tissues were rather thick, arranged as ordered rows of mainly coarse collagen fibers. The germinal layer of periosteum contained less fiber content and was rich in blood vessels and cells. In CPT tissues, the periosteum structure is drastically different, with a large number of well-differentiated fibroblasts and abundant collagen fibers that appeared disorganized (**Figure 1**). The density of blood vessels as indicated by the index of MVD was lower in CPT periosteum than the control (**Table 1**), with blood vessel wall thickening and lumen narrowing or stenosis (**Figure 2A**). The bone in CPT tissues overall was structurally disorganized. Osteoclasts and osteoblasts were distributed around bone trabecula. Fibrous connective tis-

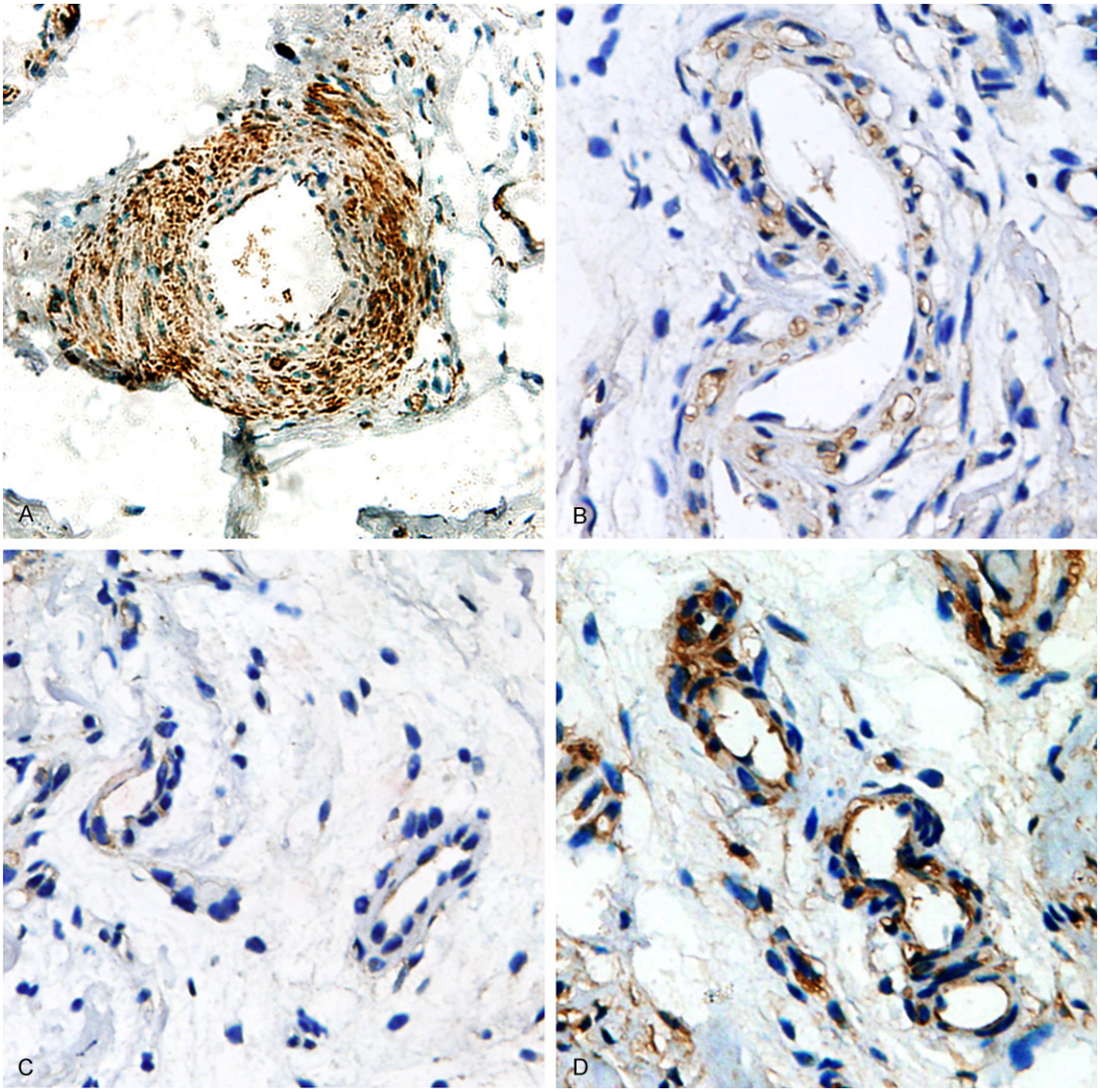

Figure 3. A. NPY expression in a blood vessel of CPT periosteum (400 ×). NPY positive structures indicated by the dark-brown color were densely stained in periosteal vascular wall of the CPT group; B. NPY expression in a blood vessel in normal periosteum; C. VIP expression in CPT periosteal blood vessel (400 ×). VIP positive particles were indicated by the pale-yellow color. The intensity of staining decreased significantly in CPT periosteal vessel wall; D. VIP expression in normal periosteal blood vessel.

sues were randomly scattered in bone tissues, with visible cartilage and osteonecrosis products (**Figure 2C**).

Immunohistochemical staining of tissue slices

Upon immunohistochemical staining, only the areas of the tissues containing NPY or VIP expression exhibited brown coloration. The main areas with positive NPY expression are the arterial wall of periosteum and surrounding periosteum (**Figure 3A**). In osteoblasts and osteoclasts, there was no NPY expression. The main areas with VIP expression were the vein wall of periosteum and the surrounding areas (**Figure 3C**), the osteoblasts (**Figure 4A**) and the osteoclasts (**Figure 4C**).

NPY and VIP staining in images of the immunohistochemical staining were quantified through two indices, MOD and MPA as indicated in Materials and Methods and the differences of the neuropeptide expression between the CPT and normal (control) groups were compared.

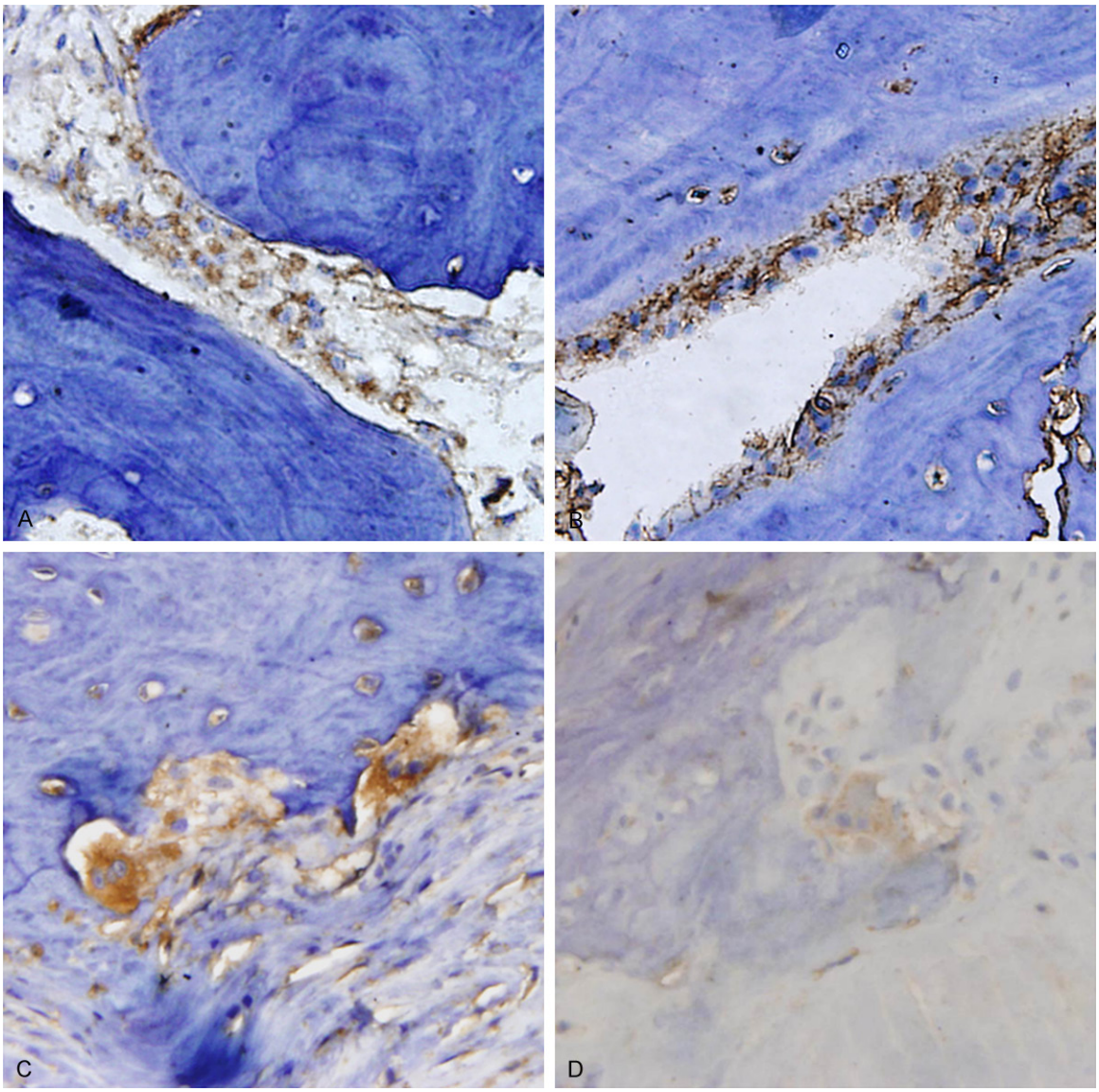

Figure 4. A. VIP expression in osteoblasts in CPT tissues (400 ×). Brown particles indicated VIP staining. VIP expression was visible in cytoplasm of osteoblasts in trabecula and surrounding areas; B. VIP expression in osteoblasts in normal tissues (400 ×); C. VIP expression in osteoclasts in CPT tissues (400 ×). Strong VIP expression indicated by the intensely dark-brown color was visible in osteoclasts surrounding trabecula; D. VIP expression in osteoclasts in normal tissues (400 ×).

NPY was expressed in both normal and CPT periosteum. The expression level was higher in the vascular wall of CPT periosteum and surrounding tissues than those of the normal tissues, as the staining was darker (with bigger MOD value) and the positively stained area was bigger (with higher MPA value), in comparison to the control group, with statistically significant differences ($P < 0.05$) (**Table 2**).

In comparison to VIP expression in the normal periosteum, VIP expression in the CPT periosteum was weaker, as indicated by the lighter VIP staining (smaller MOD value) and the smaller stained area (smaller MPA) (**Table 2**). The differences were statistically significant ($P < 0.05$). VIP expression in osteoblasts, either measurable by MOD or MPA, was not significantly different between the normal and CPT bone tissue ($P > 0.05$). VIP expression measured by MOD value, but not by MPA, was significantly enhanced in osteoclasts around fracture site of the CPT group, in comparison to the control group (**Table 3**). This is consistent with

Table 2. The expression of NPY, VIP in periosteum

	CPT periosteum (CPT group)		Normal periosteum (control group)	
	MPA	MOD	MPA	MOD
NPY	1791.53±79.730①	0.366±0.0208②	1439.90±61.381①	0.273±0.0246②
VIP	1227.487±71.799③	0.144±0.0188④	1488.260±52.834③	0.203±0.0298④

①, ②, ③, ④: There were statistically significant differences within each of the four groups ($P < 0.05$).

Table 3. The expression of VIP in bone

	CPT periosteum (CPT group)		Normal periosteum (control group)	
	Osteoblasts	Osteoclasts	Osteoblasts	Osteoclasts
MPA	796.652±9.884①	600.872±7.475②	802.381±9.961①	597.150±4.941②
MOD	0.220±0.0141③	0.390±0.0240④	0.216±0.0157③	0.168±0.0156④

①, ②, ③: There was no statistically significant difference within each of the three groups ($P > 0.05$). ④: There was statistically significant difference within the group ($P < 0.05$).

the result that VIP expression was increased only in the cytoplasm of osteoclasts in CPT fracture site (**Figure 4A**).

Discussion

Despite investigation and treatment of CPT for more than a century, the etiology of CPT is still poorly understood. There are numerous theories about the etiology of CPT. One theory is that neurofibromatosis or neuroinomatosis is the cause [4, 5]. However, the association of CPT with neurofibromatosis in clinical cases is far from being established. Using tissue of neurofibromatosis as the control, Konttinen et al. investigated bone histology of CPT periosteum in pseudarthrosis between the stump and stump site. It was found that the incidence of CPT has no direct correlation with neurofibromatosis, suggesting that CPT is a disease of non-neuronal origin [3]. Lei et al. reported in a histology study that periosteal ring constriction may be the cause. It was proposed that formation of constriction rings caused strong and aggressive constriction of the bone, thereby causing tapering, hardening and fracture of tibia, and occurrence of tibial nonunion and pseudarthrosis [2]. Herein, our findings of HE-stained tissue biopsy from 20 CPT cases are consistent with the findings of Lei et al. In the 20 CPT cases, the periosteal fiber layer and the vascular walls were thickened; the vessel lumen had stenosis and even total blockage. In addition, in the slices of bone tissues surrounding pathological lesions in the 20 CPT cases, there were existence of both osteoblasts and osteoclasts. And the structure of trabecula was disordered. Bone necrosis and cartilage components are visible in fracture site. Such abnormalities of CPT tissues may be caused by periosteum narrowing and compression which caused bone atrophy and blockage of new bone formation.

Furthermore, other mechanisms may exist that promote an increased bone resorption and changes of bone metabolism, thereby contributing to etiology of CPT. Neuropeptides may be such a mechanism as part of the neuro-osteogenic network to regulating bone metabolism [6]. In this study, the role of two well-studied neuropeptides, NYP and VIP, in CPT was investigated. NPY was first isolated from porcine brain by Tatemoto et al., containing 36 amino acid residues [7]. Early studies found that NPY is expressed in the peripheral and central nervous systems. Bjurholm et al. [8] in 1988 identified NPY-positive nerve fibers, which belong to the sympathetic nervous system, in bone tissue. The distribution of NPY in bone was mainly in the periosteum and surrounding blood vessels, with minor distribution in bone tissues including the epiphysis and bone marrow. The main biological function of NPY is vasoconstriction, or vessel shrinkage. Khor et al. found that NPY coexisted with norepinephrine to exert strong vasoconstrictive function and NPY receptors were identified in vascular smooth muscle cells. NPY regulated vessel functions through binding its receptors and releasing intracellular cyclic adenosine monophosphate (cAMP) as the second messenger [9]. With NPY mostly distributed in areas, including blood

vessel walls, the surrounding areas in the periosteum, the epiphyseal, and bone marrow close to growth plate, the main function of NPY in bone tissue is to adjust the blood flow in bone through the vasoconstrictive role of NPY [10]. Furthermore, a recent study reported that low doses of NPY are pro-angiogenesis, thereby relating NPY to bone vascularization [11]. Qin et al. [12] reported that distribution of NPY in bone development not only coincides with distribution of blood vessels, but also correlated positively with the expression pattern of vascular endothelial growth factor (VEGF), a pro-angiogenesis protein. This finding [12] supported that NPY is involved in the process of angiogenesis to influence bone remodeling. NPY may also directly regulate bone homeostasis by acting on bone cells such as osteoblasts to control their differentiation [13].

Our study demonstrated that in CPT and normal tissues NPY is expressed in periosteum, mainly in the vascular wall of periosteum. Its expression levels in CPT periosteum were much higher than those in control group. The expression of NPY was negative in bone tissue in both CPT and control groups. Our findings are overall consistent with the report of Bjurholm et al. [8] that NPY-positive nerve fibers is found mainly in the periosteum and surrounding blood vessels, with minor distribution in bone tissue. As NPY is a strong vasoconstrictor, whose vasoconstrictor capability is even stronger than the strong vasoconstrictor norepinepherine [14], elevated expression of NPY in the CPT lesions of periosteum may cause CPT periosteal blood vessels to have spasticity or shrinkage the long run, reducing the flow of blood in periosteal and bone tissue. Such an explanation is supported by our finding that the lumen of periosteal vessels in HE staining was narrower than that of in normal vessel and the amount of blood vessels was reduced. Zukowska et al. reported that low doses of NPY could increase angiogenesis and promote the vascularization of bone [15]. In our study, NPY positive expression in periosteum did not promote the vascularization of bone, but reduced blood vessels in periosteum, suggesting that NPY expression in bone tissue may have a negative role for bone vascularization. In light of Zukowska's report [15], our explanation is that excessive expression of NPY in CPT lesions results in local high concentrations of NPY, which inhibits vascularization of the bone. As bone blood supply is a key contributor to bone metabolism, excessive expression of NPY aggravates the already poor blood supply to the tibia in the lower 1/3 (CPT likely happens here), resulting a blood flow-restrictive and hypoxic environment. It has been reported that ischemic hypoxia promotes high level expression of basic fibroblast growth factor (bFGF), which promotes the proliferation of fibroblasts and vascular smooth muscle cells [16]. Thus in our setting, it is likely that bFGF may be expressed, causing periosteal fibroblasts proliferation, which causes fiber layer thickening and formation of constriction ring. And bFGF expression may cause vascular smooth muscle cell proliferating, which results in vessel wall thickening, lumen narrowing, thereby further exacerbating the hypoxic condition. In other word, there is a vicious cycle that led to the continual worsening of lesions. In summary, the effect of NPY in the CPT mainly alters the vascular flow by acting on blood vessels, thereby affecting the normal bone metabolism. Our future research will explore the expression of bFGF in CPT lesions to provide insight into the molecular events in CPT etiology.

VIP was first isolated from pig intestines by Said et al., containing 28 amino acid residues [17]. Further research also identified VIP in central and peripheral nervous system in human, mainly in the neurons of sympathetic ganglia and parasympathetic ganglion in peripheral neurons. VIP immunoreactive nerve fibers in bone tissues were mainly distributed in periosteum, bone marrow and epiphysis. In contrast to NPY, VIP is vasodilatory. The effect of VIP is reported to be dose-dependent, i.e., increasing with the increase of VIP concentration [18]. VIP also enhances the biological activity of vascular endothelial cells and promotes formation of blood vessels [19]. In our setting, VIP expression in CPT periosteal lesions was significantly lower than that of normal periosteum. Thus in CPT tissues, the vasodilatory effect of VIP would be much weaker and lumen narrower than those in normal tissues. This leads to reduced activity of vascular endothelial cells, decreased growth of blood vessels, reduced blood flow, which, together with NPY's function in vasoconstriction, leads to the formation of hypoxic environments, periosteal thickening, constricted ring formation, and inhibited bone growth. On the one hand, the hypoxic environ-

ments may cause the body, tissues, and cells to react in a number of ways, including redistribution of blood flow, increase of anaerobic metabolism to adapt to hypoxic environment, and stimulation of local cells to produce angiogenic factors such as VEGF to increase angiogenesis and restore the normal oxygen partial pressure [20]. On the other hand, severe or prolonged hypoxia may cause cell and tissue damage and bone necrosis. Also when oxygen partial pressure in bone tissue drops, expression of VEGF in osteoblasts will increase. VEGF can directly enhance the activity of osteoclast to promote bone resorption, thereby affecting fracture healing [21]. Such a scenario is supported by our findings that VIP expression was positive in cytoplasm of osteoblasts and osteoclasts of bone tissue both in CPT and control groups. Our data also indicated that in CPT group, there is elevated expression in osteoclasts based on the increase of MOD in osteoclasts, which indicates that local concentration of VIP is increased in osteoclasts of CPT lesions. Such localized high concentrations of VIP will enhance osteoclasts' ability of bone resorption, shift the balance between bone formation and osteolysis, resulting in bone resorption, the diaphysis tapering, or even fractures, nonunion, pseudarthrosis in lesions area. In summary, VIP not only acts indirectly on blood vessel by reducing the flow of local genetically to hinder bone growth, but also acts directly to stimulate bone resorption by affecting osteoclasts to promote the formation of the CPT.

The study herein indicated the abnormal expression of NPY and VIP in CPT tissues. Such abnormal expression may have two effects. First, the abnormal expression leads to narrowing of the lumen of blood vessels, reduction of the formation of new blood vessels, and reduction of blood flow, which leads to inhibition of blood flow and shortage of oxygen supply in periosteum issues. Second, as VIP stimulates osteoclast activity and shifts the balance between bone formation and osteolysis, the growth of bone leans toward bone adsorption. The two effects in combination influence the bone reconstruction in CPT. Therefore, our results suggests that NPY and VIP may be among the factors leading to CPT. CPT may be caused not only by periosteal lesion, but also by the metabolic abnormalities of bone growth via the actions of NPY and VIP. Our findings have many clinical implications. For example, based on our findings, before bone graft surgery is performed, it is important to remove not only periosteal lesions, but also bone lesions. Otherwise the residual tissue may release factors such as NPY and VIP, leading to CPT recurrence of the grafted bone.

Disclosure of conflict of interest

None.

Address correspondence to: Kelai Wang, Qilu Hospital, Shandong University, Jinan 250012, Shandong, China. E-mail: Ercolani@126.com

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