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NEDD4-1 and PTEN expression in keloid scarring

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ABSTRACT. Keloid scarring remains a major problem in plastic surgery. The aim of this study was to determine the expression of the *PTEN* tumor suppressor and *NEDD4-1* genes in keloid tissue and explore their effect on the formation of such scarring. Twenty keloid patients were enrolled in the study and underwent surgical removal of keloid tissue. No patient had received chemotherapy and/or radiotherapy prior to treatment. *PTEN* and *NEDD4-1* mRNA expression was detected by reverse transcription PCR, while PTEN protein expression was assessed using immunohistochemistry. Our results showed that levels of *PTEN* were significantly diminished in keloid samples ($P < 0.05$), whereas those of *NEDD4-1* did not significantly differ between keloid tissue and normal skin ($P > 0.05$). Furthermore, we found that *NEDD4-1* expression is high and inversely correlated with that of *PTEN* in keloids. Our results suggest that the PTEN/PI3K/AKT pathway may play an important role in keloid formation and reduces *PTEN* expression in such tissue. Finally, although *NEDD4-1* has previously been identified as a factor in keloid susceptibility, and the protein for which it encodes is known to degrade PTEN by catalyzing its polyubiquitylation, the detailed mechanism behind its involvement in keloid formation needs to be further studied.

Key words: *NEDD4-1*; PTEN; Pathological scar; Keloid

INTRODUCTION

The process of wound healing in humans is a complex one that relies on the interplay between multiple cell types, cytokines, and proteins. Under usual circumstances, this process results in a scar that over time flattens and softens, typically leaving little long-term impact. Aberrancies in this process exist, however, leading to various forms of excessive scarring such as hypertrophic scars and keloids (Naylor and Brissett, 2012).

Keloids are fibroproliferative tumors with no known malignant potential that occur in locations of dermal trauma or disruption. They are characterized by their extension beyond the confines of the original injury and often present with pain and pruritus. Additionally, these growths may result in cosmetic deformities and contribute to significant emotional distress (Naylor and Brissett, 2012). The exact etiology is unknown, but significant research efforts have been made (Xiaoxue et al., 2014). These investigations have demonstrated that several genetic alterations are involved in the carcinogenetic formation process and it has been suggested that a subset of these abnormalities concern the genes phosphatase and tensin homolog (*PTEN*) and neural precursor cell expressed, developmentally downregulated 4-1 (*NEDD4-1*).

The protein PTEN is a dual-specificity phosphatase that acts as a negative regulator of the phosphoinositide 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (AKT)/mechanistic target of rapamycin (mTOR) pathway (Liao et al., 2010; Kulkarni et al., 2011; Paterno et al., 2011), thus controlling a variety of processes related to cell survival, proliferation, and growth (Wang et al., 2008). The loss of PTEN function due to deletion, mutation, methylation, or decreased expression has been observed in human cancers (Ma et al., 2009; Miller et al., 2009; Yang et al., 2010) and some fibrotic diseases (White et al., 2003; Takashima et al., 2009). In addition, in idiopathic pulmonary fibrosis and liver fibrosis, both mRNA and protein levels of PTEN are suppressed (White et al., 2006; Larsson et al., 2008). Although keloids are not malignant tumors, the fibroblasts involved exhibit malignant features such as excessive proliferation, resistance to apoptosis, and atypical differentiation (Lim et al., 2006), and the mechanism behind their formation is not yet fully understood (Atiyeh, 2007). We therefore hypothesized that PTEN levels should be diminished in keloid pathogenesis.

In a genome-wide association study, Nakashima et al. (2010) identified *NEDD4* as a keloid susceptibility gene in the Japanese population. More recently, Zhu et al. (2013) used the Sequenom MassARRAY system to determine that *NEDD4* is also significantly associated with keloid susceptibility in the Han Chinese population. Recent research has shown that through the PI3K/AKT pathway, NEDD4 may promote fibroblast proliferation and invasiveness and cause loss of cellular contact inhibition (Akca et al., 2011). p27 protein, a cyclin-dependent kinase inhibitor, plays an important role in cell contact inhibition by arresting the cell cycle, while the highly expressed *NEDD4* gene may induce the transfer of p27 from the nucleus to the cytoplasm and promote its degradation (Liu et al., 2002). NEDD4 may also promote the excessive expression and accumulation in the extracellular matrix of fibroblast protein and type I collagen (Liu et al., 2013). Taken together, these results suggest that NEDD4 may promote keloid formation and development. Furthermore, NEDD4 has been found to effect the accumulation of β-catenin in the cytoplasm of fibroblasts, and to activate its signal transduction pathway. According to the same report, mass accumulation of β-catenin is found in keloid tissue (Sato, 2006; Chung et al., 2011). It can therefore be inferred from this that increased expression of *NEDD4* may be implicated in the formation and development of keloids. NEDD4-

1 is one of the most important NEDD4 isoforms. It was originally identified as an E3 ligase that ubiquitylates PTEN, and regulates the apoptosis and proliferation of tumor cells (Wang et al., 2007; Kwak et al., 2010). We therefore hypothesized that an inverse correlation between PTEN and NEDD4-1 levels may be implicated in keloid pathogenesis.

In the present study, the mRNA levels of *PTEN* and *NEDD4-1* in keloids and hypertrophic scars were measured by reverse transcription polymerase chain reaction (RT-PCR), and the expression of PTEN protein was assessed using immunohistochemistry. Our findings may provide a new target for the prevention and cure of keloid formation.

MATERIAL AND METHODS

Tissue specimens

Keloid tissues were obtained from 20 people (16 men and 4 women, 12-47 years old). No local infection or ulceration was evident and no study subject had been treated with glucocorticosteroids or radiotherapy. Twenty age- and site-matched normal skin (NS) specimens were obtained from donors during scar resection. Experimental protocols were approved by the hospital Ethics Committee and written informed consent was obtained from each donor.

RT-PCR

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), following the manufacturer protocol. Primers were designed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by the Shanghai Shenggong Genomics Institute (Shanghai, China). Primer sequences were as follows: *PTEN* forward: 5'-CAGAGCGAGGGGCATCAG-3', reverse: 5'-GCAGGAAATCCCATAGCAATAA-3', (238-bp product); *NEDD4-1* forward: 5'-GCATGTTTGCCATCCTCCCA-3', reverse: 5'-AGCCAGGCTTGCAAGAATTAG-3', (295-bp product); and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) forward: 5'-AGATCATCAGCAATGCCTCCTG-3', and reverse: 5'-ATGGCATGGACTGTGGTCATG-3', (109-bp product). Each PCR was performed as follows: denaturation at 94°C for 5 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. After 35 cycles, products of the exponential phase of the reaction were assessed to allow semi-quantitative comparison of complementary DNA synthesized from identical reactions. Amplification products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. The gray values of each bands in RT-PCR gel were analyzed by the Image J 2.1.4.7 software.

Immunohistochemistry

Keloid and NS tissues were fixed with formalin and embedded in paraffin following routine histological practices. A primary antibody against PTEN was used at a dilution of 1 in 200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To detect antibody-bound cells, an avidin/biotinylated enzyme complex was used (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Tissues were examined under an Olympus microscope (Olympus, Tokyo, Japan) and images were taken using a SPOT digital microscope camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Data analysis

Data are reported as means ± SE and were analyzed using the Shapiro-Wilk test. Statistical significance was determined using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Pearson's correlation test was also used to examine relationships, and P values equal to or less than 0.05 were considered to be statistically significant.

RESULTS

PTEN expression in keloid and NS tissues

Results were obtained for all cases. By semi-quantitative RT-PCR, we found that *PTEN* mRNA expression was markedly reduced in keloid tissue compared to that of NS (representative samples are shown in Figure 1A). Quantitative analysis from 20 patients also indicated that *PTEN* mRNA levels in keloid tissues were significantly lower than those in NS samples (Figure 1B, $P < 0.01$). In addition, immunohistochemical examination demonstrated strong PTEN immunoreactivity in both the cytoplasm and nuclei of epithelial and dermal fibroblasts in NS tissues (Figure 1C), while only a weak signal, or an absence thereof, was detected in keloid samples (Figure 1D). This suggests that PTEN protein expression is diminished in keloid tissue.

NEDD4-1 expression in keloid and NS tissues

Using semi-quantitative RT-PCR, we found that *NEDD4-1* mRNA expression did not significantly differ between keloid and NS specimens (representative samples are shown in Figure 1E). Quantitative analysis from 20 patients also indicated that *NEDD4-1* mRNA levels in keloid tissues were not significantly lower than those in NS samples (Figure 1F, $P > 0.05$.)

NEDD4-1 expression is high and inversely correlated with that of *PTEN* in keloid tissue

Although overall *NEDD4-1* expression was not found to be significantly different between in keloid tissues and NS, this gene has previously been identified as a factor resulting in keloid susceptibility, and the encoded protein is a ubiquitin ligase capable of degrading PTEN. Therefore, to investigate the correlation between *PTEN* and *NEDD4-1* levels in keloid tissues (Figure 2, lane 1), hypertrophic tissues (Figure 2, lane 2) and normal tissues (Figure 2, lane 3), RT-PCR analysis was performed using specific primers against these two transcripts (Figure 2). A significant inverse correlation between *PTEN* and *NEDD4-1* expression was confirmed.

In summary, quantitative analysis of samples from 20 patients showed that the level of *PTEN* was significantly lower in keloid tissue than in NS.

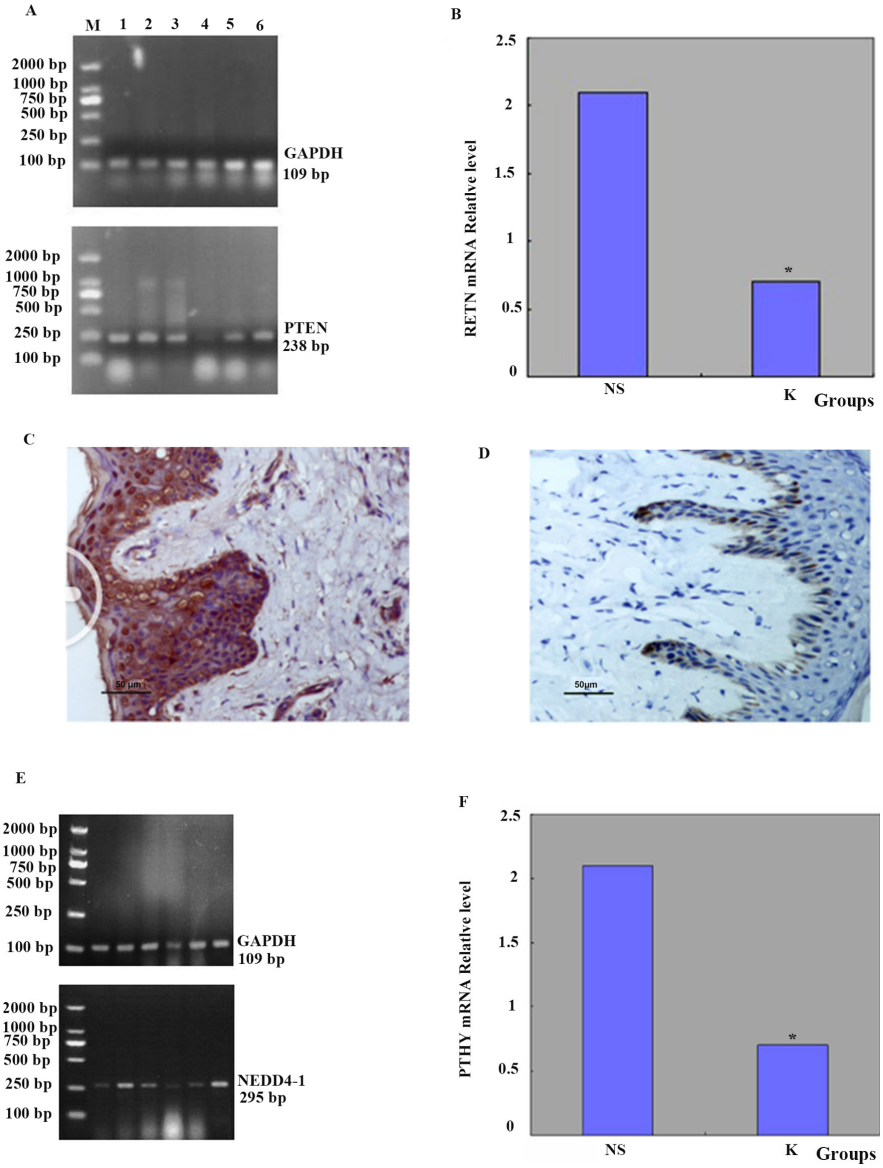

Figure 1. *PTEN* and *NEDD4-1* expression in tissues. **A.** Reverse transcription polymerase chain reaction (RT-PCR) analysis of *PTEN* mRNA in tissues from two patients. *Lane M*, DNA marker. **B.** Semi-quantitative evaluation of *PTEN* mRNA level in two tissue types. Data are reported as the ratio of *PTEN* expression to that of *GAPDH*. NS, normal skin; K, keloid tissue. *P < 0.05. **C.** Immunohistochemical staining of PTEN in NS. Strong PTEN immunoreactivity (brown particles) was observed in the cytoplasm of epithelial and dermal fibroblasts in NS. Bar = 50 µm. **D.** Immunohistochemical staining of PTEN in keloid tissue, showing very little immunoreactivity. Brown particles indicate a strong reaction. **E.** RT-PCR analysis of *NEDD4-1* mRNA in tissues from two patients. *Lane M*, DNA marker. **F.** Semi-quantitative evaluation of *NEDD4-1* mRNA level in two tissue types. Data are reported as the ratio of *NEDD4-1* expression to that of *GAPDH*. NS, normal skin; K, keloid tissue. *P > 0.05.

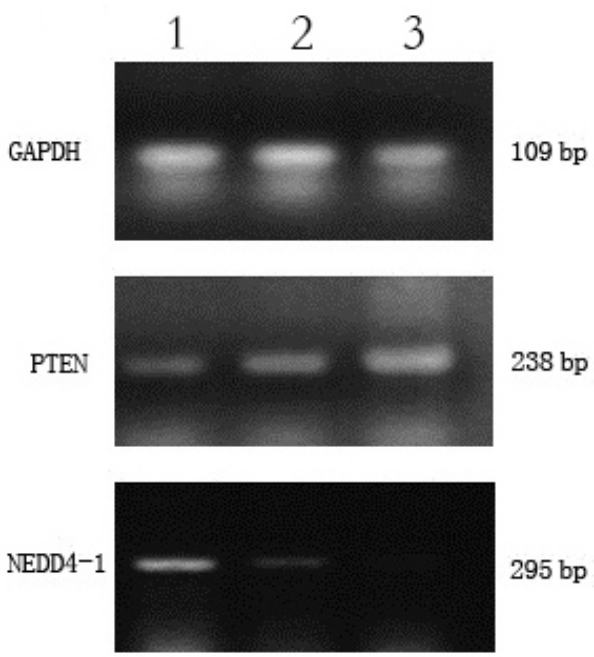

Figure 2. Reverse transcription polymerase chain reaction analysis of PTEN and NEDD4-1 expression in keloid tissues (*lane 1*), hypertrophic tissues (*lane 2*) and normal tissues (*lane 3*). GAPDH served as a loading control.

DISCUSSION

Keloids (Xiaoxue et al., 2008) are benign skin tumors that may appear after wound healing in genetically predisposed patients. They are characterized by excessive proliferation of fibroblast cells and an overabundance of collagen at the site of a healed injury (Al-Attar et al., 2006), and can invade skin beyond the boundaries of the original wound, with no spontaneous regression (Fujiwara et al., 2005).

It has been generally accepted that loss of PTEN function with subsequent activation of the PI3K/AKT signaling pathway contributes to carcinogenesis and fibrotic diseases (Li et al., 2012), and PTEN has been proposed as an effective anti-fibrosis target (Xia et al., 2008; Parapuram et al., 2011).

In the present study, we found that *PTEN* mRNA expression was significantly downregulated in keloid tissue compared to NS. Furthermore, immunohistochemical analyses revealed that the level of PTEN protein was also lower in samples derived from keloid specimens. These results suggest that the expression of *PTEN* is downregulated at both transcriptional and translational levels in keloids, and abnormal *PTEN* expression is involved in their pathogenesis. In conclusion, loss of PTEN function may play an important role in the development of keloid tissue.

The ubiquitin-mediated proteasomal pathway is an important mechanism that regulates protein stability. Recently, Wang et al. (2007) found that the level of PTEN protein is

regulated by ubiquitin-mediated proteosomal degradation (Figure 1), and identified its ubiquitin ligase as NEDD4-1. NEDD4-1 degrades PTEN protein by catalyzing PTEN polyubiquitylation. In cells, overexpression of *NEDD4-1* promotes Kirsten rat sarcoma viral oncogene homolog (K-RAS)-induced oncogenic cellular transformation in a *PTEN*-dependent manner. Moreover, in human urinary bladder cancers, an inverse relationship between NEDD4-1 and PTEN levels has been observed (Wang et al., 2007). Together, these data strongly suggest that NEDD4-1 demonstrates oncogenic activity by suppressing PTEN function. To date, the expression and cellular localization of NEDD4-1 has not been established in keloid scarring. To extend our knowledge regarding the role of NEDD4-1 in this process, we compared its expression in keloid and normal tissue.

Although expressed in both tissue types, no significant difference in the level of *NEDD4-1* was observed between keloid and NS samples. In addition, we also found that *NEDD4-1* expression levels were inversely correlated with those of *PTEN* in keloid tissues, indicating its proto-oncogenic properties.

In addition to its oncogenic function, NEDD4-1 is believed to play a role in tumor suppression by catalyzing PTEN monoubiquitylation, leading to PTEN nuclear import (Trotman et al., 2007), where it plays a crucial role in maintaining chromosomal integrity (Shen et al., 2007; Trotman et al., 2007).

NEDD4-1 regulates PTEN function, and may play a crucial role in the development of keloids. However, expression of *NEDD4-1* mRNA was not seen to differ significantly between keloid and normal tissue in this study. There are several possible reasons behind this finding. First, recent research into the genetics of keloid development has shown that this condition is a polygenic disease, and may be influenced by the additive effect of several susceptibility genes, as well as environmental factors. Taken on its own, a single susceptibility gene will tend to have a minor impact and may not affect the overall process of keloid development, as appears to be the case with *NEDD4-1*. Second, this result may be related to selection bias due to a relatively small sample size. Third, the formation of keloids may be caused by mutations in the *NEDD4-1* gene that do not affect the level of protein expressed, and thus in that regard, significant differences between keloid and normal tissue would not be detected.

In humans, the *NEDD4-1* gene is expressed in both normal skin tissue and keloids. In terms of its being implicated in susceptibility, the role of *NEDD4-1* in the occurrence and development of keloid tissue requires further study. In the future, two aspects need to be thoroughly addressed. First, experimental error and selection bias can be reduced by increasing the sample size. Second, as *NEDD4-1* sequence variations caused by genetic mutations presumably exist, any differences in this respect can be contrasted by experimental detection and database search and alignment. In addition, advanced exome sequencing technology may present a new method for further inquiry into keloid susceptibility genes.

Conflicts of interest

The authors declare no conflict of interest.

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