Vol. 36 / No. 1 / April 2024
Sinta 2 (Decree No: 200/M/KPT/2020)
Online ISSN : 2549-4082 ; Print ISSN : 1978-4279
Available online at https://e-journal.unair.ac.id/BIKK

Berkala Ilmu Kesehatan Kulit dan Kelamin

Literature Review
Periodical of Dermatology and Venereology

Epigenetic Alterations in Keloid a Possible Method to Find Novel Agents for Keloid Treatment

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ABSTRACT

Background: Keloids are dermal fibro-proliferative disorders due to prolonged wound healing processes with excessive collagen depositions, which produce symptoms of itching and pain, cosmetic disfigurement, and limitation of joint motion. Standard treatment for keloid has not been accepted yet. It may be due to the complexities and poorly understood keloid development that are driven by various factors from systemic to local, genetic to epigenetic. Since genetic factors are difficult to manipulate, an approach to epigenetic factors may be hopeful. **Purpose**: To review various related reports on epigenetic factors such as DNA methylations, histone modifications, and micro-RNAs, which have significant roles in keloid development and can be used as targets for novel agents in keloid treatment. **Review:** Various genes in keloid fibroblasts (KFs) are repressed by DNA methylation, and one of them can inhibit the regulation of TGF-β1/Smad signaling, whereas another gene may influence anti-fibrotic events. Either inhibitor of methyl-transferase, inhibitor of histone-acetyltransferase, or histone-deacetylase can reduce TGF-β1/Smad signaling in KFs. Abnormal expressions of pro-fibrotic miRNAs have been identified in KFs and transfection KFs with anti-fibrotic miRNAs such as miRNA-205 and miRNA- 31, evidently can inhibit VEGF signaling. Furthermore, transfection of miRNA-637 into KFs can inhibit KFs in proliferation, migration, and collagen synthesis through TGF-β1/Smad signaling. Apoptosis and cellular senescence in KFs can also be stimulated by miRA-34 and miRNA-30. **Conclusion**: In the future, targets in epigenetic events such as inhibitors of methyl-transferase, histone-acetyltransferases, and histone-deacetylases, together with various miRNA, may be applied as novel agents for the treatment of keloid.

Keywords*:* Keloid, keloid fibroblast phenotypes, epigenetic factors, new candidate agents.

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| Article into |
Submited: 13-12-2023, Accepted: 16-02-2024, Published: 31-03-2024

BACKGROUND

Keloids are dermal fibro-proliferative disorders due to abnormal-prolong cutaneous wound healing with excessive deposition of collagen[1], and they are clinically diagnosed by finding extended scars into the surrounding skin to perform clawing edges or pseudopodia[2]. Keloids can not only produce symptoms of itching and pain, but they can also cause obvious cosmetic disfigurement and limitations in joint motion[3]. All of these may affect the patient's quality of life, similar to psoriatic patients, when they are compared with healthy controls[4]. Another cross-sectional survey on the burden of keloid disease showed that having keloids was associated with considerable impairment of emotional well-being[5].

Keloid tissues are composed of heterogenic tissues with stiffness and range from soft to extremely firm with decreased skin plasticity. The center tissue of the keloid often exhibits central hypoxia due to capillary occlusion as a result of exuberant collagen deposition[6]. The margins of keloid contain active fibroblasts that invade surrounding tissue and are well-vascularized[7]. These vascular components are thought to be a result of angiogenesis due to endothelial cell migration under stimulatory various growth factors[8]. Several studies have shown that the outer margins and inner part of keloids are populated by different types of stem cells, where hematopoietic-mesenchymal stem

DOI : 10.20473/bikk.V36.1.2024.60-67

cells are located in the outer margin and surrounding tissues[9].

In recent years, various medical and surgical methods have been used to treat keloids, but these interventions have not reached optimal results. The recurrence rate is still high, and until now, there has been no accepted method as the gold standard for keloid treatment[10,11]. This condition may be caused by complexities and poorly understood keloid development. It may be driven by multiple factors, including genetic and epigenetic ones, which one another collaborates to cause persistent inflammation, and continue to stimulate keloid fibroblast activities, and prevent scar maturation[12]. Various manipulations based on skin tension and local risk factors have been tried to treat and prevent keloid recurrences, such as site-specific treatment protocols as suggested by the Japan Scar Workshop Consensus Document 2018[13], but the results are still unsatisfactory.

Although various techniques in biotechnology are available now, the genetic factors of keloids are still difficult to manipulate. The hope may lie in epigenetic events. Various inhibitors of keloid epigenetic factors can be isolated. These materials can be considered candidate materials for the new treatment of keloid.

This article reviews various related reports on epigenetic factors such as DNA methylations, histone modifications, and micro-RNAs (miRNAs), which have significant roles in keloid development and can potentially be used as targets for novel agents in keloid treatment.

REVIEW

Phenotypes and genotypes of keloid fibroblasts. Collagen architecture the distance and thickness of bundles in keloid tissue is significantly different compared to normal skin, normal and hypertrophic scar[14]. These phenomena are related to excessive collagen synthesis and a low degree of collagen degradation. In collagen synthesis, transforming growth factor β1 (TGF-β1) /Smad signaling is an important factor. Various studies indicated that keloid fibroblasts (KFs) are highly sensitive against TGF-β stimulation in collagen synthesis, cellular proliferations and cellular migration[15]. Another study showed that KFs can up-regulate TGF-β1 expression by autocrine signaling. The mechanism of TGF-β1/Smad signaling on KFs is mediated by mitogen-activated protein kinase pathways, including the extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 pathways[16].

Keloid fibroblasts are also up-regulating vascular endothelial growth factor (VEGF) expression[16]; this may be secondary to stimulation of hypoxia-inducible factor-1 (HIF-1) which is expressed under hypoxia due to excessive collagen accumulation in keloid tissues. VEGF on keloid tissues is not only stimulating angiogenesis, but it also stimulates collagen deposition[17]. In addition, collagen accumulation depends not only on collagen synthesis but also on collagen degradation by an active matrix metalloproteinase (MMP)[8]. MMP activities can be inhibited by tissue inhibitor matrix metalloproteinases (TIMPs) which are found at a higher level in keloid tissue compared to either normal skin or normal scars[18]. The activation of MMP needs plasmin and can be inhibited by plasminogen activator inhibitor (PAI). A study showed that PAI expression is elevated among KFs[19], and this elevation is responsible for VEGF signaling through ERK signals[20].

The renin-angiotensin system (RAS), which was formerly well known in pathogenesis of hypertension and renal damage, also plays significant roles in dermal fibrosis. RAS components such as angiotensin II (Ang II), angiotensin-1 (AT1) receptor, angiotensin -2 (AT2) receptor, and angiotensin-converting enzyme (ACE) are also expressed in the skin and act independently from the plasma RAS. The concentration of the AT1 receptor is higher in patients with keloid than in the hypertrophic scar and normal skin[21]. The effect of elevated levels of angiotensin II (Ang II) on the AT1 receptor contributes to increased expression of VEGF and TGF-β1 and connective tissue growth factor (CTGF), while at the same time suppressing the inhibitors of anti-fibrotic tissues of matrix metalloproteinase (TIMPs)[22]. In addition, mast cells's chymase is able to stimulate fibroblast proliferations and migrations, together with enhancing the expression of angiotensin II, collagen I, TGF-β1, and interleukin-1β among KFs[23].

In normal wound healing, fibroblasts eventually become senescent as a control mechanism for fibroblast proliferation and extracellular matrix synthesis. Among keloids, KFs exhibit decreased apoptosis rates, due to their characteristic resistance to Fas-mediated apoptosis and in defective cellular senescence due to increasing telomerase activity[25]. In this way, both defective cellular senescence and resistance against apoptosis may contribute to high proliferation rates of KFs and invasion of KFs into surrounding skin, since as mentioned above, KFs from the margin of keloid tissues can proliferate better than KFs from central ones.

A study of 22,284 genes isolated from normal fibroblasts and KFs showed that 43 genes were overexpressed and five genes were underexpressed in keloid fibroblasts. Among the overexpressed genes, a few of them are tumor-related genes[26]. Another study found that some of the genes of KFs are related to multiple fibrosis-associated pathways, such as up-regulating the expression of several insulin-like growth factor (IGF)-binding and IGF-binding-related proteins and down-regulating the expression of a subset of wingless-related integration (Wnt) pathway inhibitors and multiple IL-1-inducible genes[27].

*Epigenetic factors in keloid fibroblasts***.** The term "epigenetics" has been formally defined for decades as a change in the state of expression of a gene (or a trait) that does not involve a mutation, but that is nevertheless inherited (at least through a mitotic division) in the absence of the signal or event that initiated the change. Another term "epigenomics" has been widely adopted operationally by the research community to indicate studies on a genome-wide level. Epigenetic studies focus on the analysis of DNA methylation, histone modifications, and noncoding micro-RNAs[28].

*DNA methylation in keloid***.** The most common epigenetic modification among keloids is DNA methylation; it refers to a chemical modification process in which a methyl group is transferred to a carbon atom in the cytosine ring of the DNA molecule by an enzyme named DNA methyltransferase (DNMT). This process mostly happens in CpG dinucleotides. When methylation occurs in the promoter region of a gene, transcription of this gene will be repressed.

Studies among KFs indicated that DNMT is 100% expressed, which differs from normal fibroblasts, which express only 8%[29]. It indicates that DNA methylations are frequent and have significant roles in keloid development. By using linear models for microarray data, among KFs, it was revealed that of the 100,000 differentially methylated CpG sites compared with normal fibroblasts, 20,695 of them are found to be hypomethylated and 79,305 are hypermethylated[30]. Targeted promoter regions for methylation are different between keloid tissue and normal skin[31]. Adding a methyl transferase inhibitor (5-aza-2-deoxycytidine) into KFs culture can suppress the expression of TGF-β1, phospho-smad 2 and phospho-smad 3, and inhibit KFs proliferation[32]. It means there are various genes repressed by DNA methylation, and their products may have an affect on the inhibition of the upregulation of TGF-β1/Smad signaling among KFs. Those genes have not been identified yet, but one of them may be on anti-fibrotic gene, namely Thy-1. A study among cardiac fibroblasts indicated that hypoxia can stimulate DNMT expression because there are putative hypoxia-responsive elements on the promoters of all three active DNMTs [33], and culturing human lung fibroblasts in hypoxia conditions can stimulate methylation on the promoter region of the anti-fibrotic gene Thy-1, together with the silencing of this gene[34].

Excessive collagen synthesis together with low-level collagen degradation may affect KFs phenotype through hypoxia. In addition, hypoxia can stimulate angiogenesis by upregulating various genes, one of which is VEGF gene[35]. VEGF is well known for its ability to stimulate collagen synthesis among KFs[17]. The relationship between excessive collagen synthesis, hypoxia, methylation of anti-fibrotic genes, and VEGF gene among KFs has never been discovered.

Histone modifications in keloid. Histone proteins act as spools around which DNA winds and packs into chromosomes, and their modifications act in various events such as packing DNA into chromosomes, activation-inactivation of transcriptional DNA, and repairing or damaging DNA. In other words, histone modifications can influence gene expression without any effect on their sequences. Activation of gene transcription begins with histone modification in the form of histone acetylation, which is mediated by histone acetyltransferases (HAT), and transcriptional activity is suppressed by histone deacetylation, which is mediated by histone deacetylases (HDACs).

Publication about histone modification of keloid is not as common as other epigenetic events, especially studies about miRNA. Rusell et al. (2010) reported that there are various altered fibrotic-associated gene expressions among KFs. One of them is the Wnt inhibitor secreted frizzled-related protein 1 (SFRP1). Differ with decreasing expression of fibrotic-associated genes such as matrix metalloproteinase 3 and dermatopontin that are due to DNA methylation; decreasing SFRP1 expression among KFs is associated with decreased histone acetylation[36].

As mentioned above, TGF-β1/Smad signaling has important roles in collagen deposition by KFs in keloid tissues. Among fibrotic human diseases, TGF-β1/Smad signaling has also been considered a pro-fibrotic signal[37] and an in vitro study indicates that TGF-β1/Smad signaling can be inhibited by using HDACs. Using inhibitors of HATs and HDACs for specific fibrotic signals may be benefit for preventing and treating fibrotic human diseases[38].

Micro-RNA on keloid. MiRNAs are a class of small, noncoding RNAs that range from 18 to 25 nucleotides in length and modify gene expression by binding to target messenger RNA (mRNA), causing degradation of the target mRNA, or inhibiting the translation into proteins. The human genome may encode over 1900 miRNAs, although more recent analysis indicates that the number is closer to 600 miRNAs[39]. Various studies show that pro-fibrotic miRNAs and anti-fibrotic miRNAs have been identified[40]; some of them may be expressed abnormally among keloid cells and have significant roles in keloid development[41].

Functional annotations of differentially expressed miRNA targets revealed that they were enriched in several signaling pathways important for scar formation in wound healing. A study of miRNA expression among keloid epidermis showed that the expression of miRNA-513a, miRNA-374b, miRNA-21, miRNA-146, miRNA-3653, miRNA-4649, and miRNA-3156 is significantly higher compared to the epidermis of normal skin[42]. Those miRNAs may play a signature in the epithelial-mesenchymal transition on keloid keratinocytes. In addition, a study to compare miRNA expressions between keloid tissues and normal ski using miRNA microarray analysis has identified 32 differentially expressed miRNAs, 23 of which exhibited higher expression, while 9 miRNAs demonstrated lower expression in keloid tissue[43].

Micro-RNAs related to VEGF expression**.** As mentioned before, VEGF is upregulated among KFs as a secondary effect of HIF-1 expression. VEGF is not only responsible for angiogenesis in marginal sites of keloid, but VEGF is also responsible for collagen deposition in keloid tissues by inhibiting collagen degradation by stimulating TIMP and PAI expression. Various miRNAs are related to VEGF expression and signaling; one of them is miRNA-205. It was first studied among human glioma cell lines and functioned as a tumor suppressor[44]. Transfection of KFs by miRNA-205-5p showed that this miRNA can suppress VEGF expression by inhibition the PI3K/Akt pathway[45].

Another miRNA with a similar effect on VEGF expression is miRNA-31. MiRNA-31 expression is increased among keloid tissues and KFs; downregulation of miRNA-31 can inhibit cellular proliferation and cell cycles and induce apoptosis of KFs by mediating the HIF-1/VEGF signaling pathway[46].

Micro-RNAs related to TGF-β1. Excessive collagen synthesis, cellular proliferation, and migration of KFs are related to TGF-β1/Smad signaling. KFs are not only highly sensitive against TGF-β1 stimulation, but they also act as TGF-β1 producer. Various reports have shown that the TGF-β pathway among various human cells needs an important component in its downstream signaling cascades, and its various miRNAs pathway[47].

TGF-β signaling has been shown to modulate miRNA expression at both the transcriptional and post-transcriptional levels, and several miRNAs have been experimentally validated to be modulators of TGF-β signaling at multiple levels by targeting ligands, receptors, R-Smad, co-Smad, I-Smad, and non-Smad pathway components as well as downstream targets of TGF-β signaling[48]. One of the miRNAs that has a suppression effect on Smad signaling is miR-637. This miRNA is lowly expressed among the epidermis of keloid tissues, and transfection of this miRNA onto KFs culture can inhibit Smad 3 signaling with suppression of KFs proliferation rates and inhibition in cellular invasion[49]. Another epigenetic factor with contrary effects on TGF-β1 signaling and its expression of KFs is miRNA-21, which can stimulate KFs proliferation, and trans-differentiation and produce enzymes that are needed for invasion into normal skin[50]. Inhibition of miRNA-21 activity among keloid tissues may affect KFs phenotypes

Micro-RNAs related to apoptosis and cellular senescence of keloid fibroblasts. Keloid fibroblasts exhibit a low rate of apoptosis because they are resistant to fas-mediated apoptosis. They also have high telomere activity, which is as a sign that it is difficult for them become senescent. It has been known that fas-mediated apoptosis is upregulated by p53[51].

Recently, it has been shown that components of the p53 pathway are direct targets of various micro RNAs, and their overexpression leads to the induction of apoptosis, cellular senescence, and cell cycle arrest. One of them is miRNA-34[52]. This miRNA can modulate the apoptotic gene expression of KFs, as it was found when KFs are treated with ingenol mubutate[53], a natural component that is considered a novel agent for treating keloid[54].

Apoptosis is well known to have the capacity to be inhibited by BCL-2, a protein whose expression can be upregulated by miRNA-21[55] and downregulated by miRNA-30a[56]. A study indicates that miRNA-30a expression among keloid tissues is low, but its expression can be elevated by giving trichostatin-A, an HDAC inhibitor with various target genes[57]. Trichostatin A is not only known to stimulate the apoptosis of KFs, but it can also suppress collagen synthesis by KFs[58].

Keloid fibroblasts are characterized by defective cellular senescence processes due to increasing telomerase activity25, but this phenotype is only found among KFs in the keloid margin. Based on β-galactosidase staining and proliferation rate analysis, KFs from the deep and center keloid tissues indicate that their cellular senescence and cell cycle are arrested[59]. Several miRNAs, including miR-106b, miR-125b, miR-126, miR-146a, miR-21, miR-22, miR-29, miR-210, miR-34a, miR-449a, miR-494, and miR-17-92 cluster and miR-200 family, are differentially expressed in senescent cells or aged tissues and play a role in cellular senescence[60], but unfortunately the expression of those miRNAs on KFs, except for miRNA-21, has never been studied. MiRNA- 21 is well known for its anti-senescent effects on KFs by down-regulating smad 7, and therefore it up-regulates TGF-β1/smad signaling[61]. Electron beam radiations, which are already described as one of the modalities in keloid treatment, are capable of stimulating KFs senescence by inhibiting miRNA-21 activities[62]. Similar to this, ultraviolet-B (UVB) exposure can stimulate KFs senescence by inhibiting KFs cellular viabilities, collagen deposition, and TGFβ-1 production[63].

Micro-RNAs related to Renin Angiotensin system. As described above, the Renin Angiotensin System (RAS) also has significant roles in dermal fibrosis and keloid, and the effect of Ang II on the AT1R contributes to increased expression of VEGF and TGF-β1 and connective tissue growth factor (CTGF) among KFs together with suppressing the inhibitors of anti-fibrotic tissues of TIMPs. Among keloid lesions, those activities are performed by embryonic stem cells like populations that are located in specific areas of keloid, namely keloid-associated lymphoid tissues[64], and they express vitamin D receptor and renin-angiotensin[64,65].

A study indicates that renin mRNA overexpression among people with hypertension is under regulation by various miRNAs; one of them is miRNA-181[66], and now it is considered a miRNA that contributes to RAS overactivity together with elevation of blood pressure[67]. Unfortunately, studies about this miRNA expression, either in keloid tissues or KFs, have never been performed yet.

DISCUSSION

Experimental researches on KFs or keloid tissues will determine new agents for keloid treatment in the future. Although various techniques in biotechnology are available now, genetic factors of keloids are still difficult to manipulate. The hope may lie in epigenetic events. Various inhibitors in keloid epigenetic factors can be isolated. These materials can be considered as candidate materials in the new treatment of keloid.

Based on the literature review above, TGF-β1/Smad signaling on KFs that is responsible for KFs proliferation, migration, and collagen synthesis, can be inhibited by giving methyl transferase inhibitors such as 5-aza-2-deoxycytidine, or inhibitor of histone-acetyl transferase, or histone-deacetylases, or miRNA-637.

In addition, due to the action of miRNA 21 in TGF-β1/smad signaling, searching for inhibitors of miRNA 21 and finding its effects on KFs or keloid tissues is merely important. MiRNA-31 and miRNA-205 may be potential new agents to treat keloids in the future. The role of those miRNAs in the activation of MMP and collagen degradation among KFs or keloid tissues is also important to be clarified.

Stimulating KFs of keloid tissues to suffer cellular senescence and apoptosis by clinical application of various methods such as using various miRNAs, trichostatin A, and UVB irradiation is also important to be researched. Furthermore, since the Renin Angiotensin System (RAS) has an important role in keloid development and varies miRNA involved in RAS activities, finding miRNAs involved in RAS activities among KFs is important to field research in the future.

In the future, targets in epigenetic events such as inhibitors of methyl-transferase, histone-acetyltransferases, and histone-deacetylases, together with various miRNAs, may be applied as novel agents for the treatment of keloid

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