Integrated microRNA-mRNA network analysis of invasive and immune-inflammatory pathways in sebaceous carcinoma of the eyelid with pagetoid spread

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Abstract. Pagetoid spread is a poor prognostic factor in sebaceous carcinoma (SGC) of the eyelid; however, its underlying molecular mechanisms remain unclear. The present study aimed to investigate the expression profiles of microRNAs (miRNAs/miRs) and mRNAs in SGC of the eyelid with pagetoid spread, compared with cases without pagetoid spread, and to explore potential miRNA-mRNA regulatory networks. Total RNA was extracted from three patients with SGC of the eyelid with pagetoid spread (pagetoid group), three patients without pagetoid spread (non-pagetoid group), and three patients with normal tarsal plate tissue obtained during free tarsal plate reconstruction (control group). Small RNA sequencing was performed using a next-generation sequencer to identify differentially expressed miRNAs among the groups, and bioinformatics analyses of mRNA expression profiles were conducted to determine associated biological functions and construct miRNA-mRNA interaction networks. In the pagetoid group, upregulated mRNAs and downregulated miRNAs were associated with enhanced invasion, migration and immune-inflammatory responses. Three miRNAs, miR-1275, miR-1976 and miR-330-5p, were commonly involved in these functional networks. Conversely, downregulated mRNAs and upregulated miRNAs were associated with impaired lipid metabolism, including miR-760-3p, miR-1266-5p, miR-3918, miR-1269a and miR-198. These findings suggest that SGC of the eyelid with pagetoid spread demonstrates increased invasive and migratory capacity, reduced lipid metabolic function and enhanced immune-inflammatory responses, which may contribute to its aggressive biological behavior.

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Introduction

Sebaceous carcinoma (SGC) of the eyelid is a malignant tumor arising from the meibomian or Zeiss glands (1). SGC of the eyelid is highly prevalent in Asia and represents the most common eyelid malignancy (accounting for 43.7% of cases in Japan) (2). The tumor is generally resistant to radiotherapy and chemotherapy, with complete surgical resection remaining the mainstay of treatment. However, postoperative local recurrence and lymph node metastasis have been reported in 6-24 and 8-21% of patients, respectively, reflecting a relatively high-grade malignancy (3-6). Adjuvant and combination therapies may improve prognosis; however, the pathogenesis of SGC of the eyelid remains unexplored, and sufficient research to guide diagnosis and treatment has not been performed.

Several clinical and pathological factors have been identified as poor prognostic indicators and risk factors for postoperative recurrence in SGC of the eyelid. Clinical factors include increased tumor diameter (6-8), tumor localization (6,9), and delayed therapeutic intervention due to misdiagnosis at initial presentation (10). Pathologic factors include diffuse growth patterns, multicentric tumor origin, non-lobular pattern, and poor differentiation (11-14). In addition, pagetoid spread is a critical poor prognostic factor. It is characterized by diffuse intraepithelial dissemination and a tendency to invade the cornea and conjunctiva, complicating complete surgical excision. Furthermore, it is frequently misdiagnosed as chronic blepharoconjunctivitis, contributing to the unfavorable prognosis (12,15-21).

Recent advances in genetic analyses have provided insights into the biological pathways involved in the tumor microenvironment and pathogenesis of SGC. Whole-genome studies (22-25) and microRNA profiling (26-28) have been performed to elucidate tumor biology. These studies have revealed recurrent genetic alterations and dysregulated miRNA expression profiles associated with tumor proliferation, invasion, and immune-related pathways in SGC.

Increasing evidence suggests that immune-inflammatory pathways play a critical role in tumor invasion and progression by shaping the tumor microenvironment (29,30). In various malignancies, dysregulated immune responses and immune-related signaling pathways have been implicated in epithelial-mesenchymal transition, tumor invasiveness, and metastatic potential (29,30). In sebaceous carcinoma, recent genomic and transcriptomic studies have identified dysregulated immune- and inflammation-related pathways associated with aggressive tumor behavior and invasion (22-28). These findings suggest that immune-inflammatory mechanisms may contribute to the invasive behavior of SGC and provide a biological rationale for investigating immune-related molecular networks in this disease.

In recent years, integrated bioinformatic approaches combining miRNA and mRNA expression data have been widely applied to various malignancies to elucidate regulatory networks underlying tumor invasiveness and therapeutic resistance, as well as to identify potential diagnostic and prognostic biomarkers (29,30).

Despite these advances, controlling local recurrence and lymph node metastasis remains challenging, and clinical outcomes are often suboptimal. In particular, limited genetic research has focused on the pathogenesis of pagetoid spread, a major determinant of poor prognosis in SGC.

Our previous study published in 2021 (28) primarily focused on global miRNA expression profiling in eyelid sebaceous carcinoma. In contrast, the present study uniquely applies an integrated miRNA-mRNA network analysis to specifically explore the molecular mechanisms underlying pagetoid spread, with particular emphasis on invasion-related and immune-inflammatory pathways.

Therefore, this study aimed to elucidate the genetic mechanisms involved in SGC of the eyelid with pagetoid spread.

Materials and methods

Patient and tissue samples. Nine patients who underwent surgical treatment for eyelid tumors at Toyama University Hospital were included in this study. These patients underwent surgical treatment between January 2020 and October 2024. Cases were selected based on the availability of sufficient tumor tissue for molecular analyses and a confirmed pathological diagnosis by histopathological examination. Based on pathological findings, the patients were divided into three groups, each comprising three participants: SGC of the eyelid with pagetoid spread, SGC of the eyelid without pagetoid spread, and normal controls. The pagetoid spread group included an 88-year-old woman, a 74-year-old woman, and an 81-year-old man. The maximum tumor diameters in this group were 6, 7, and 8 mm, respectively, and all tumors were classified as T1 according to the American Joint Committee on Cancer (AJCC) staging system (8th edition). Regarding histopathological growth patterns, one case in the pagetoid spread group (a 74-year-old woman) showed a diffuse growth pattern, whereas the remaining two cases showed a nodular growth pattern. The non-pagetoid spread group consisted of an 84-year-old woman, a 69-year-old woman, and an 80-year-old man. The maximum tumor diameters and T classifications in this group were 10 mm (T1), 6 mm (T1), and 11 mm (T2b), respectively. All cases in the non-pagetoid spread group exhibited a nodular growth pattern. No cases in either group showed evidence of multicentric origin based on clinicopathological evaluation. Normal control samples were obtained from a 72-year-old woman, a 74-year-old woman, and a 77-year-old man, from whom normal tarsal plate tissue was collected during free tarsal plate reconstruction. This study was approved by the Institutional Review Board of the University of Toyama (approval no. R2015051), and all procedures were conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients prior to enrollment.

RNA extraction and quality control. Total RNA was extracted from whole tumor tissue samples stored at -80˚C after surgical excision, without prior formalin fixation. Tumor-predominant areas were selected as much as possible based on pathological evaluation; however, RNA was extracted from whole tissue sections without manual macrodissection, and adjacent non-neoplastic tissues may have been partially included. Total RNA, including miRNA, was extracted from tissue samples using the NucleoSpin miRNA kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's protocol. RNA quality and quantity were assessed using a Bioanalyzer 2100 system with the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA) (31).

Library preparation and RNA sequencing (mRNA and miRNA). Comprehensive mRNA and miRNA analyses were subcontracted to OligoAtenta, Inc. (Tokyo, Japan) and performed using their next-generation sequencing (NGS) service. For library preparation, Illumina-compatible kits were used, and sequencing was conducted with either paired-end or single-end reads on the NovaSeq 6000 system (Illumina, San Diego, CA, USA).

For mRNA analysis, mRNA was selectively extracted and purified from total RNA. Sequencing libraries were subsequently prepared and analyzed using 150 bp paired-end reads. The resulting FASTQ data were processed using a standard pipeline provided by OligoAtenta, which included adapter removal, quality assessment, read mapping, and expression quantification. Reads were mapped to the human reference genome (GRCh38), and gene expression levels were normalized using transcripts per million (TPM) or fragments per kilobase of transcript per million mapped reads.

In the miRNA analysis, small RNA libraries were prepared, and reads of 83 nucleotides in length were obtained. Each read contained a unique molecular identifier (UMI) and a 3' adapter sequence, which were automatically trimmed during data processing. Clean reads were mapped to miRbase, and UMI-based deduplication was performed to obtain expression data normalized by reads per million. Additional processing steps included adapter removal (N) using Trim Galore, genome mapping with Bowtie2, file conversion via Samtools, and annotation analysis using Strand NGS software. The raw RNA sequencing data generated in the present study have been deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under BioProject accession number PRJDB40390.

Raw read data processing. The first 50 bp sequences of each raw 150 bp sequence read were extracted using Seqkit. Adaptor

sequences were subsequently trimmed from the 50 bp reads using Cutadapt. Low-quality (Q score <20) and short reads (<10 bp) were removed using the FASTX-Toolkit. The filtered reads were then aligned to hg19, and miRNA annotation was performed using Strand NGS version 3.3.

Integrated miRNA-mRNA interaction analyses. To examine the molecular functions and interaction networks of expressed miRNA and mRNA, combined data from the present study and previously published datasets generated by our group were analyzed using Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA).

Results

Identification of expressed miRNAs and mRNAs. Before molecular analyses, clinicopathological characteristics were reviewed to confirm the comparability of the study groups. All pagetoid spread cases were classified as pathological T1 according to the AJCC staging system (8th edition); one case exhibited a diffuse growth pattern, whereas all remaining cases in both the pagetoid and non-pagetoid groups showed a nodular growth pattern, and no case in either group showed evidence of multicentric origin. A total of 280,241,626 raw reads were obtained for the entire SGC of the eyelid dataset, with each sample yielding at least 20 million reads. This sequencing depth was sufficient to ensure reliable miRNA and mRNA expression analyses. Quality assessment was performed on the raw sequencing data. Low-quality reads and adapter sequences were removed from the datasets. For miRNA analysis, the filtered reads were aligned to the human miRBase using Strand NGS and mapped to known miRNAs. For mRNA analysis, quality-filtered reads were similarly mapped to the human reference genome (GRCh38) using the STAR aligner. The transcript expression levels were quantified as TPM. Expression variation and functional analyses were subsequently performed on both datasets. miRNAs with at least a 1.5-fold change and mRNAs with at least a 2.0-fold change in expression relative to control samples were identified. The Venn diagram in Fig. 1 illustrates the number of genes with increased expression. Fig. 1A presents the number of upregulated mRNAs, whereas Fig. 1B depicts the number of downregulated miRNAs known to suppress the expression of their target genes. Compared with the control group, 789 and 2,205 mRNAs were upregulated in the non-pagetoid and pagetoid groups, respectively, of which 62 were uniquely upregulated in the pagetoid group. By contrast, 2,298 and 1,940 miRNAs were downregulated in the non-pagetoid and pagetoid groups, respectively, of which 1,025 were uniquely downregulated in the pagetoid group. The Venn diagram in Fig. 2 illustrates the number of genes with decreased expression. Fig. 2A and B present the numbers of downregulated and upregulated mRNAs, respectively. Compared with the control group, 667 and 187 mRNAs were downregulated in the non-pagetoid and pagetoid groups, respectively, with 40 uniquely downregulated in the pagetoid group. Conversely, 3,179 and 2,640 miRNAs were upregulated in the non-pagetoid and pagetoid groups, respectively, of which 1,239 were uniquely upregulated in the pagetoid group.

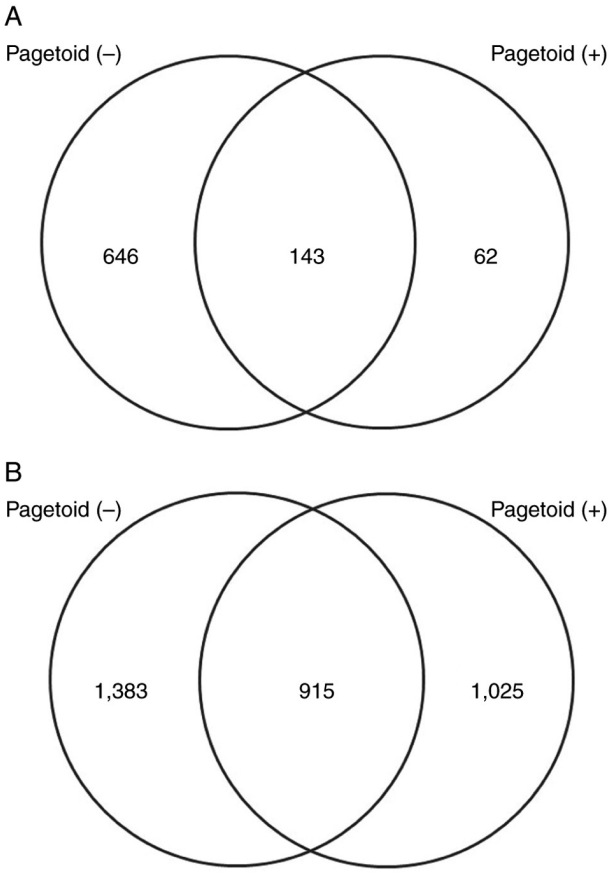

Figure 1. Venn diagrams of differentially expressed mRNAs and microRNAs in SGC classified by pagetoid spread status. (A) mRNAs with ≥2-fold upregulation compared with controls. (B) microRNAs with ≥1.5-fold downregulation compared with controls. The diagrams illustrate unique and shared differentially expressed genes or microRNAs between pagetoid-positive and pagetoid-negative SGC groups. SGC, sebaceous gland carcinoma.

Functional analyses of expressed miRNAs and mRNAs. To investigate the biological functions and canonical pathways associated with pagetoid spread, an integrated miRNA-mRNA dataset comprising 62 upregulated mRNAs and 1,025 downregulated miRNAs specifically identified in the pagetoid group was analyzed using IPA software. Consequently, biological functions with positive Z-scores (indicating predicted activation) were predominantly associated with tumor cell infiltration and migration (e.g., cell movement, migration, and invasion of tumor cell lines) and with immune responses (e.g., proliferation and activation of leukocytes and lymphocytes). The top five biological functions in each category are presented in Tables I and II. An integrated dataset consisting of 40 downregulated mRNAs and 1,239 upregulated miRNAs specifically observed in the pagetoid group was subsequently analyzed. IPA analysis predicted the inhibition of biological functions with negative Z-scores, particularly those related to lipid metabolism, including lipid synthesis and fatty acid metabolism. The top five inhibited biological functions are presented in Table III.

Construction of molecular interaction networks of expressed miRNAs and mRNAs. To further elucidate regulatory interactions between miRNAs and mRNAs in the pagetoid group,

Table I. Top 5 biological functions (excluding immune- and inflammation-related functions) associated with differentially expressed miRNAs (|fold changel ≥1.5) and mRNAs (|fold changel ≥2.0) in eyelid sebaceous gland carcinoma with pagetoid spread.

Functional annotation	P-value	Predicted activation state	Activation z-score	No. of molecules
Cell movement of tumor cell lines	$5.03x10^{-6}$	Increased	5.635	54
Migration of tumor cell lines	$9.67x10^{-6}$	Increased	5.106	50
Invasion of tumor cell lines	$3.78x10^{-6}$	Increased	4.761	48
Invasion of cells	$1.93x10^{-6}$	Increased	4.735	53
Cell movement	$1.01x10^{-11}$	Increased	4.67	96

Differentially expressed miRNAs and mRNAs were identified based on predefined fold-change thresholds (≥1.5 for miRNAs and ≥2.0 for mRNAs). No statistical hypothesis testing was applied at the individual gene level. P-values shown in this table represent enrichment significance calculated using Fisher's exact test within Ingenuity Pathway Analysis. Activation z-scores were used to predict functional activation or inhibition.

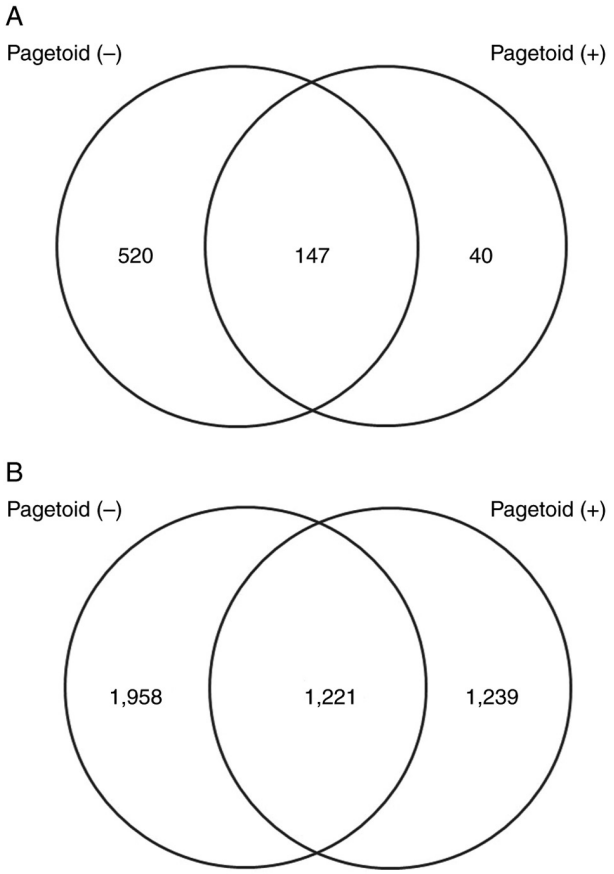

Figure 2. Venn diagrams of differentially expressed transcripts of mRNAs and microRNAs in SGC classified by pagetoid spread status. (A) mRNAs with ≥2-fold downregulation compared with controls. (B) microRNAs with ≥1.5-fold upregulation compared with controls. The diagrams depict unique and shared differentially expressed transcripts between pagetoid-positive and pagetoid-negative SGC groups. SGC, sebaceous gland carcinoma.

target prediction analysis was performed using the miRNA Target Filter tool in IPA. Consequently, two miRNA-mRNA networks associated with increased gene expression were identified: i) The invasion and migration pathway (Fig. 3) and ii) the immune-inflammatory response pathway (Fig. 4). Notably, both networks shared three common target miRNAs: miR-330-5p, miR-1275, and miR-1976. These miRNAs were consistently involved in gene expression regulation in the pagetoid group and functioned as hub nodes, playing central roles within the networks. Moreover, a miRNA-mRNA network associated with lipid metabolism and gene expression suppression was identified (Fig. 5). This network analysis suggested that five miRNAs, miR-760-3p, miR-1266-5p, miR-3918, miR-1269a, and miR-198, were involved in the regulation of lipid metabolism.

Discussion

Complete surgical resection is the standard treatment for SGC of the eyelid; however, the risk of recurrence remains relatively high even when surgery is performed with an adequate safety margin (3-6). Intraepithelial invasion through pagetoid spread is a major factor contributing to local recurrence (15-19). Therefore, elucidating the pathogenesis of SGC of the eyelid with pagetoid spread, exploring adjuvant or combination therapies alongside surgery, and developing diagnostic biomarkers are essential for improving patient outcomes. In the present study, the expression profiles of miRNAs and mRNAs specifically associated with SGC of the eyelid with pagetoid spread were analyzed. Integrated miRNA-mRNA datasets were analyzed to investigate the biological functions, canonical pathways, and miRNA-mRNA networks associated with clinicopathological features. Consequently, SGC of the eyelid with pagetoid spread exhibited upregulated expression of genes related to invasion, migration, and immune-inflammatory responses, organized into interconnected regulatory networks. These networks appear to be epigenetically regulated by miR-330-5p, miR-1275, and miR-1976. Furthermore, downregulated expression of genes involved in lipid metabolism was observed, with related networks regulated by miR-760-3p, miR-1266-5p, miR-3918, miR-1269a, and miR-198.

Several studies have examined miRNA expression in SGC. The downregulation of miR-200c and miR-141, which regulate the expression of ZEB1, a transcription factor involved in epithelial-mesenchymal transition, has been associated with SGC malignancy (32). ZEB2 has been identified as a

Table II. Top 5 immune- and inflammation-related biological functions associated with differentially expressed miRNAs (|fold change| ≥1.5) and mRNAs (|fold change| ≥2.0) in eyelid sebaceous gland carcinoma with pagetoid spread.

Functional annotation	P-value	Predicted activation state	Activation z-score	No. of molecules
Leukopoiesis	$1.58x10^{-20}$	Increased	4.241	58
Cytotoxicity of leukocytes	$6.6x10^{-16}$	Increased	4.204	23
Activation of leukocytes	$2.52x10^{-28}$	Increased	4.19	63
Hematopoiesis of mononuclear leukocytes	$2.42x10^{-19}$	Increased	4.104	51
Cytotoxicity of lymphocytes	$2.35x10^{-15}$	Increased	4.088	22

Biological function analysis and activation z-scores were generated using IPA. P-values represent enrichment significance calculated using Fisher's exact test within IPA. Functions were ranked according to their activation z-scores. miRNA, microRNA; IPA, Ingenuity Pathway Analysis.

Table III. Top 5 inhibited biological functions, particularly those related to lipid metabolism, associated with differentially expressed miRNAs (|fold change| ≥1.5) and mRNAs (|fold change| ≥2.0) in eyelid SGC with pagetoid spread.

Functional annotation	P-value	Predicted activation state	Activation z-score	No. of molecules
Transport of molecule	0.00783	Decreased	-3.291	25
Fatty acid metabolism	0.000301	Decreased	-2.202	15
Transport of lipid	0.00429	Decreased	-1.331	7
Synthesis of lipid	0.00318	Decreased	-1.15	16
Absorption of lipid	0.000598	Decreased	-1.123	4

Differentially expressed miRNAs and mRNAs were identified based on predefined fold-change thresholds (≥1.5 for miRNAs and ≥2.0 for mRNAs). Biological function analysis and activation z-scores were generated using IPA. P-values represent enrichment significance calculated using Fisher's exact test within IPA. Functions were ranked according to their activation z-scores. miRNA, microRNA; IPA, Ingenuity Pathway Analysis.

target of miR-651-5p, and its expression is regulated by this miRNA (33). Furthermore, miR-3907 promotes proliferation and migration in SGC (34). Reduced expression of miR-518d and miR-211, both of which suppress cell proliferation, has also been associated with SGC (27). Additionally, miR-205 and miR-199a have been implicated in the maintenance of cancer stemness (26). To date, only one study has specifically investigated miRNAs involved in SGC of the eyelid with pagetoid spread. That study demonstrated that, compared with the nodular type, the pagetoid type exhibited over-expression of miR-205 and downregulation of miR-199a, with corresponding overexpression of their respective target genes EZH2 (regulated by miR-205) and CD44 (regulated by miR-199a) (26). CD44 overexpression is recognized as a marker of cancer stemness and has been reported to promote cell adhesion to the extracellular matrix and migration through epithelial-to-mesenchymal transition, thereby activating adhesion, migration, and proliferative signaling pathways (35). These findings are consistent with the present study, in which invasion- and migration-related pathways were upregulated. In our previous work, miR-146a-5p, miR-149-3p, miR-193a-3p, miR-195-5p, and miR-4671-3p were identified as miRNAs involved in the proliferation of SGC, whereas miR-130a-3p and miR-939-5p were associated with the suppression of lipid metabolism (28). Thus, SGC is regulated by miRNAs that promote proliferation, enhance invasion and migration through epithelial-mesenchymal transition, and suppress lipid metabolism.

Consistent with these previous findings, the present study further demonstrated that SGC of the eyelid with pagetoid spread exhibits greater invasive and migratory potential and a more pronounced reduction in lipid metabolism compared with the non-pagetoid type.

SGC with pagetoid spread was found to form specific gene networks involving key molecules that promote cancer cell metastasis, invasion, and proliferation. These molecules include signal transducer and activator of transcription 1 (STAT1) (36,37), glial cell line-derived neurotrophic factor (38,39), interferon regulatory factor 7 (40), retinoic acid-inducible gene I (40), poly (ADP-ribose) polymerase family member 9 (41,42), granzyme B (43), and interleukin-1 receptor-associated kinase 1 (44).

Furthermore, SGC with pagetoid spread forms immune-inflammatory networks, which contribute to the recruitment and migration of immune cells, such as lymphocytes and monocytes, and may be involved in chronic inflammation, immune evasion, and tumor immunity. The molecules involved in this network include CC chemokine ligand 5 (45), cyclic GMP-AMP synthase (46), STAT1 (47), interleukin-12 receptor β1 subunit (48), hepatitis A virus cellular receptor 2 (49), C-X-C motif chemokine ligand 3 (50), signaling threshold regulating transmembrane

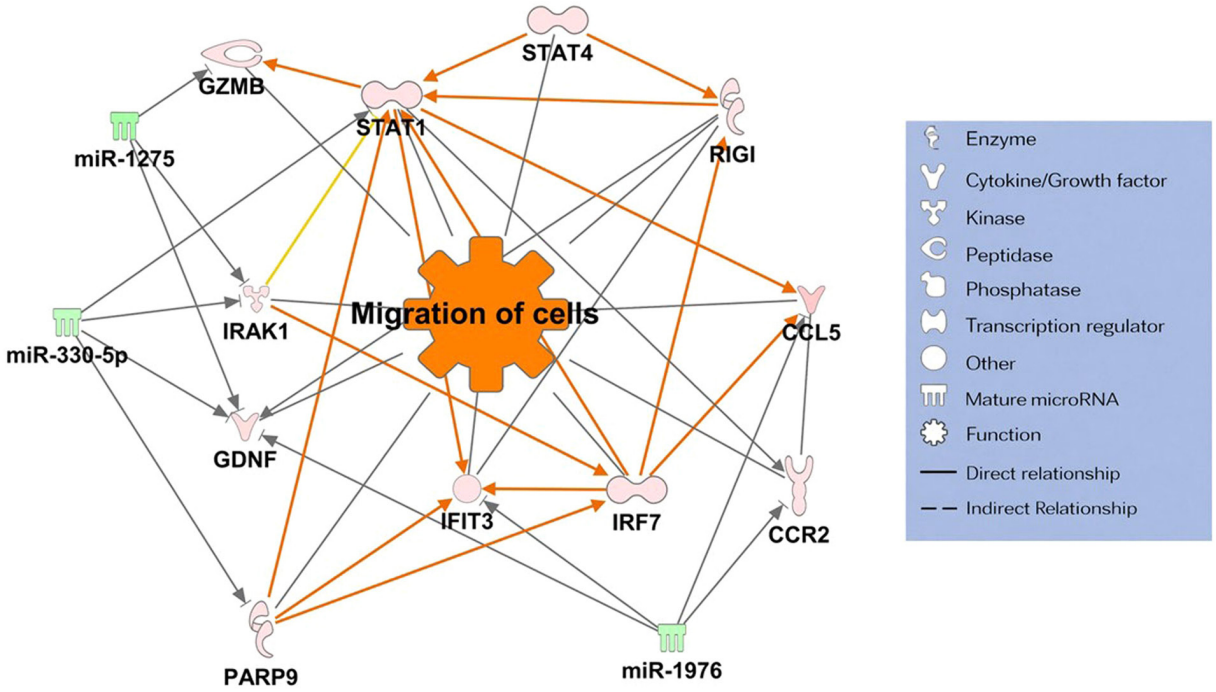

Figure 3. Molecular network of cell migration-related genes regulated by three downregulated microRNAs and eleven upregulated mRNAs. The network illustrates the interactions between three downregulated microRNAs and eleven upregulated mRNAs involved in the biological function 'migration of cells'. Predicted or validated microRNA-mRNA interactions are shown, indicating potential regulatory relationships contributing to enhanced cell motility.

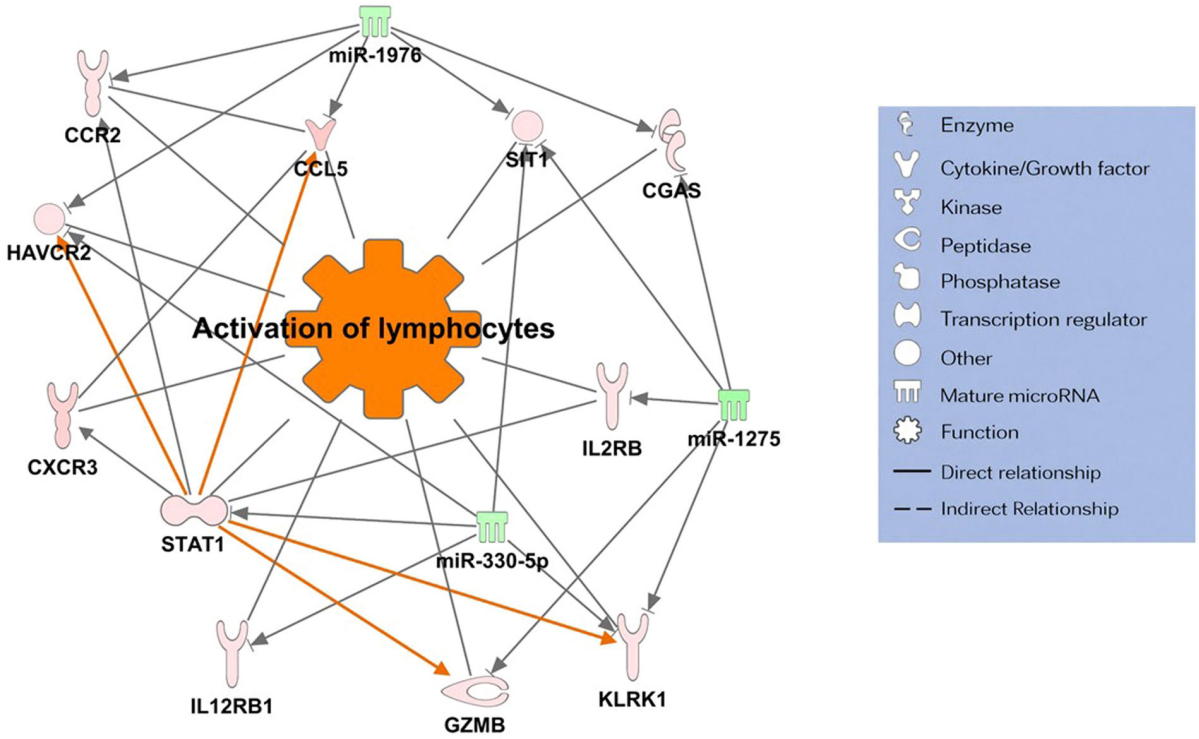

Figure 4. Molecular network related to lymphocyte activation involving three downregulated microRNAs and eleven upregulated mRNAs. The figure depicts a regulatory network associated with the biological function 'activation of lymphocytes'. The network includes three downregulated microRNAs and eleven upregulated mRNAs, illustrating predicted or validated miRNA-mRNA interactions that may contribute to enhanced lymphocyte activity.

adaptor 1 (51), and granzyme B (43). Notably, both the invasion/proliferation-related and inflammation-related gene networks were commonly regulated by three downregulated miRNAs: miR-330-5p, miR-1275, and miR-1976. miR-1275 is downregulated in head and neck, colorectal, and esophageal cancers, where it regulates cancer cell migration, invasion, and proliferation (52-54). In pancreatic cancer, it also plays a key role in natural killer cell function and immune evasion (55).

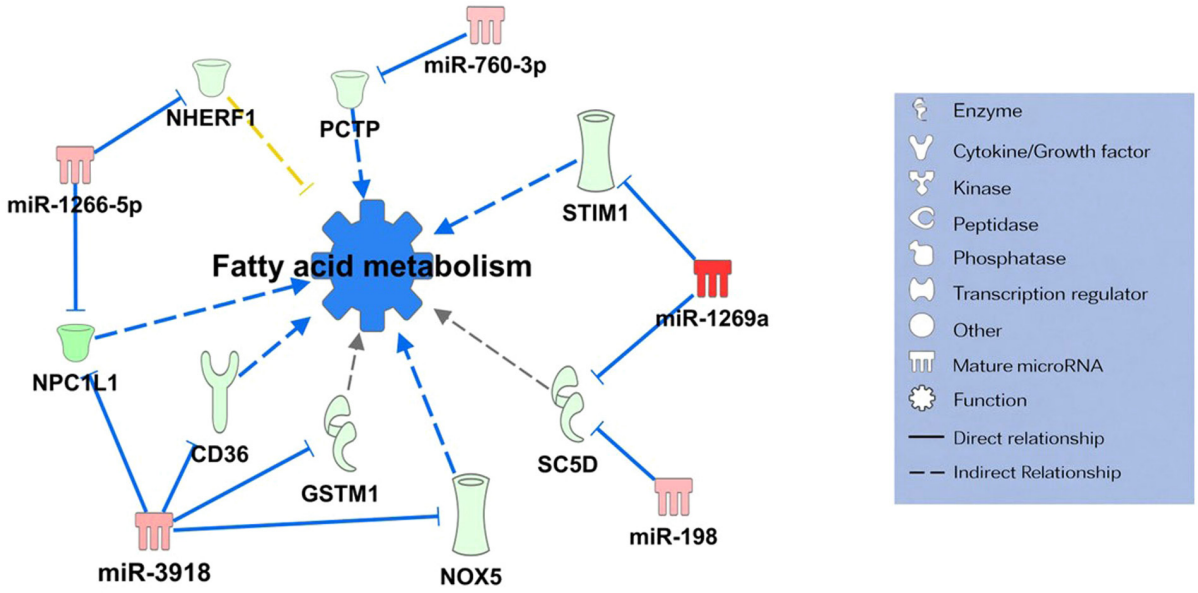

Figure 5. Molecular network related to fatty acid metabolism involving five upregulated microRNAs and eight downregulated mRNAs. The figure illustrates a molecular network associated with the biological function 'fatty acid metabolism'. The network includes five upregulated microRNAs and eight downregulated mRNAs, depicting predicted or experimentally supported miRNA-mRNA interactions that may play a role in the regulation of lipid metabolic processes.

miR-1976 is downregulated in breast and non-small cell lung cancers, and its decreased expression has been associated with the promotion of epithelial-mesenchymal transition (56,57). In ovarian cancer, it has been implicated in the regulation of tumor-infiltrating immune cells (58). Similarly, miR-330-5p is downregulated in non-small cell lung and papillary thyroid cancers, where it promotes cell proliferation, migration, and invasion (59,60). In ovarian cancer, it regulates antigen presentation by tumor cells and immune cell infiltration (61). Thus, the downregulation of miR-330-5p, miR-1275, and miR-1976 are thought to play key epigenetic roles in regulating invasion, migration, and inflammatory immune networks in SGC with pagetoid spread.

These invasion- and migration-related networks may not be specific to pagetoid spread alone but could also reflect other established clinicopathological risk factors, such as tumor size and invasive growth patterns. Likewise, the immune-inflammatory networks identified may be related to tumor-infiltrating lymphocytes, which are recognized as important biological features of SGC.

SGC with pagetoid spread exhibits suppressed lipid metabolism and forms distinct gene networks. Intracytoplasmic lipid accumulation in SGC can be detected by immunohistochemical staining for adipophilin or Oil Red O staining, both of which are recognized as pathological markers of SGC (62,63). Abnormal lipid metabolism in sebaceous glands contributes to the development of SGC and represents a key factor in understanding its pathogenesis. In a previous study, lipid metabolism was suppressed in SGC compared with sebaceous adenoma (28). The present findings suggest that SGC of the eyelid with pagetoid spread is associated with a more pronounced dysregulation of lipid metabolism-related pathways than the non-pagetoid type, which may be related to its higher malignant potential. The gene network related to lipid metabolism includes sterol-C5-desaturase (64), cluster of differentiation 36 (65), phosphatidylcholine transfer protein (66), Niemann-Pick C1-like 1 (67), glutathione S-transferase M1 (68), NADPH oxidase 5 (69), stromal interaction molecule 1 (70), and Na+/H+ exchanger regulatory factor 1 (71). The downregulation of this gene network was regulated by miR-760-3p, miR-1266-5p, miR-3918, miR-1269a, and miR-198. These miRNAs are suggested to function as upstream regulators of genes involved in lipid metabolism in SGC with pagetoid spread.

This study has several limitations. First, the small sample size precluded stratified or multivariate analyses adjusting for established high-risk clinicopathological factors, such as tumor size, pathological T stage, diffuse growth pattern, and multicentric origin. Although tumor size, T stage, and multicentricity were carefully documented, the molecular differences observed in this study may not be exclusively attributable to pagetoid spread alone and could be influenced by overlapping pathological features. Second, the miRNA-mRNA interactions identified in this study were based on integrated expression analyses and bioinformatic predictions derived from clinical tumor specimens. Direct experimental validation of miRNA binding to the 3' untranslated regions of target genes, such as dual-luciferase reporter assays or functional assays using cell line models, was not performed. Accordingly, the proposed regulatory relationships should be considered putative, and further functional studies using appropriate experimental models will be required to confirm direct miRNA-mRNA interactions and their biological effects. Third, although immune-inflammatory pathways were identified at the transcriptomic level, no histopathological or immunohistochemical validation of the tumor immune microenvironment was performed. In particular, the expression of immune checkpoint molecules such as PD-L1 and the extent of tumor-infiltrating lymphocytes, including $CD8^+$ T cells, were not evaluated in this study. Therefore, the immune-related findings should be interpreted as transcriptional alterations, and future studies incorporating immunohistochemical analyses will be required to clarify their pathological and clinical significance.

In addition, this study was not designed as a direct experimental comparison between pagetoid and nodular subtypes of SGC. Rather, it aimed to explore molecular features associated with pagetoid spread using integrated miRNA-mRNA expression analyses; therefore, the identified molecular networks should be interpreted as pagetoid-associated signatures rather than definitive subtype-specific determinants. Taken together, this study should be regarded as an exploratory, hypothesis-generating analysis identifying pagetoid-associated miRNA-mRNA regulatory networks in clinical SGC specimens. These findings provide a conceptual framework for future mechanistic and translational studies, including functional validation in experimental models and comprehensive clinicopathological correlation analyses.

This study provides the first comprehensive characterization of miRNA-mRNA interaction networks in SGC of the eyelid with pagetoid spread. Several miRNAs with altered expression were identified, potentially regulating critical functional changes, including enhanced invasion, migration, and inflammatory responses, and suppressed lipid metabolism. These findings advance the current understanding of the pathophysiological mechanisms underlying SGC of the eyelid and may aid in the identification of potential biomarkers and therapeutic targets. Further experimental validation and detailed investigation are required to elucidate the precise functional roles of these miRNA-mRNA networks associated with pagetoid spread. Future studies are expected to clarify how these networks contribute to improved diagnostic and therapeutic strategies for SGC.

Acknowledgements

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Availability of data and materials

The data generated in the present study may be found in the DNA Data Bank of Japan Sequence Read Archive under BioProject accession number PRJDB40390 or at the following URL: https://ddbj.nig.ac.jp/resource/bioproject/PRJDB40390. The dataset is also accessible via the NCBI BioProject database at the following URL: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJDB40390.

Authors' contributions

TY conceived and designed the study, performed the experiments and drafted the manuscript. TH, YF and YT contributed to the experimental work and data acquisition. AH contributed to the conception and design of the study, and to the interpretation of the data. AH and YT supervised the project and provided critical revision of the manuscript. AH and YT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the University of Toyama (approval no. R2015051). All procedures were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients prior to surgery, including consent for participation in the study and the use of resected specimens for research purposes.

Patient consent for publication

Written informed consent for publication was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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