Next-generation sequencing of the basal cell carcinoma miRNome and a description of novel microRNA candidates under neoadjuvant vismodegib therapy: an integrative molecular and surgical case study

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Received 29 September 2015; accepted 27 October 2015

Background: MicroRNAs (miRNAs) have been identified as key players in posttranscriptional gene regulation and have a significant impact on basal cell carcinoma (BCC) development. The Sonic hedgehog pathway inhibitor vismodegib has been approved for oral therapy of metastatic or advanced BCC. Here, a high-throughput miRNA sequencing analysis was carried out to identify differentially expressed miRNAs and possible novel miRNA candidates in vismodegib-treated BCC tissue. Additionally, we described our surgical experience with neoadjuvant oral vismodegib therapy.

Patients and methods: A punch biopsy (4 mm) from a patient with an extensive cranial BCC under oral vismodegib and a corresponding nonlesional epithelial skin biopsy were harvested. Total RNA was isolated, after which a sequencing cDNA library was prepared, and cluster generation was carried out, which was followed by an ultra-high-throughput miRNA sequencing analysis to indicate the read number of miRNA expression based on miRBase 21. In addition to the identification of differentially expressed miRNAs from RNA sequencing data, additional novel miRNA candidates were determined with a tool for identifying new miRNA sequences (miRDeep2).

Results: We identified 33 up-regulated miRNAs (fold change ≥2) and 39 potentially new miRNA candidates (miRDeep scores 0–43.6). A manual sequence analysis of the miRNA candidates on the genomic locus of chromosome 1 with provisional IDs of chr1_1913 and chr1_421 was further carried out and rated as promising (chr1_1913) and borderline
(chr1_421). Histopathology revealed skip lesions in clinically healthy appearing skin at the tumor margins, which were the cause of seven re-excisions by micrographic controlled surgery to achieve tumor-free margins.

**Conclusion:** miRNA sequencing revealed novel miRNA candidates that need to be further confirmed in functional Dicer knockout studies. Clinically, on the basis of our surgical experience described here, neoadjuvant vismodegib therapy in BCC appears to impede histopathologic evaluations with effects on surgical therapy. Thus, larger studies are necessary, but are not preferable at this time if other options are available.

**Key words:** miRNAs, basal cell carcinoma, noncoding RNAs, next-generation sequencing

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**introduction**

Basal cell carcinoma (BCC) is the most frequently occurring tumor in humans, accounting for 80% of nonmelanoma skin cancers [1, 2]. Although micrographic controlled surgery with cleared tumor margins remains the treatment of choice in selective advanced cases when surgical therapy is not possible or in metastatic disease, therapeutic options were previously limited to radiation or systemic chemotherapy with 5-fluorouracil and cisplatin. Vismodegib is the first FDA-approved (January 2012) oral Sonic hedgehog (SHH) inhibitor for patients with locally advanced or metastatic BCC [3]. BCCs show molecular–pathological changes in the activation of the SHH signal transduction pathways [4, 5]. The pharmaceutical effect of vismodegib is caused by modulating SHH pathways. SHH is a ligand in the hedgehog family and binds to the patched (PTCH1) receptor. PTCH1 has a limiting effect on the smoothened (SMOH) protein, which is naturally present on the cell membrane [6]. SMOH limits the expression of the zinc finger transcription factor Gli1, which counteracts cell-cycle arrest and induces the development of hair follicle tumors [7–10]. Loss-of-function mutations of the PTCH1 receptor were shown in 70% of BCCs [7, 11, 12]. On the other hand, gain-of-function mutations of SMOH have been demonstrated in 10% of all BCCs.

MicroRNAs (miRNAs) are a group of 17–23 nt short, non-coding RNAs with pivotal roles in posttranscriptional gene regulation [13, 14]. The characterization of miRNA machinery and the expression of miRNAs themselves have received considerable attention in tumor biology and nonmelanoma skin cancer research [15–19]. The majority of available studies are microarray and RT-PCR-based, and were undertaken to identify differentially expressed miRNAs in BCC [20, 21]. However, next-generation sequencing (NGS) technology has rapidly advanced and offers the advantage of quantifying miRNA expression by quantifying the number of reads per miRNA and simultaneously identifying novel miRNA sequences not annotated in miRBase [22]. Although the general role of miRNAs in tumor formation has begun to be examined, recent studies have provided strong evidence for the participation of miRNA dysregulation in BCC development. The miRNA expression of nodular and infiltrative BCCs was compared in a sequencing study by Heffelfinger et al. [19], who showed 20 miRNAs with significant differential expression in BCC subtypes. Sonkoly et al. [23] described miR-203 as a tumor suppressor in a BCC mouse model, which was suppressed by activation of the SHH signal transduction pathway. Our group also described a set of 16 significantly up-regulated and 10 significantly down-regulated miRNAs in a microarray-based profiling study based on miRBase 16 [20].

The present case study was carried out with an integrative approach combining our surgical experience with molecular biological and bioinformatical methods with several goals.

The number of miRBase-annotated miRNAs is constantly growing and has reached 1881 miRNA precursors and 2588 mature miRNA sequences annotated in miRBase version 21. The expression of miRNAs in vismodegib-treated BCC tissue has, to our knowledge, not been analyzed. First, we aimed to describe differentially expressed miRNAs based on miRBase 21, which is the most updated version of the database, in vismodegib-treated BCC. In addition, we wished to identify possible new miRNA candidates in BCC through a computational biology approach with the miRDeep algorithm, which scores the compatibility of the position and the frequency of sequenced RNA with the secondary structure of the miRNA precursor by applying RNA sequencing. This enables a more precise miRNA expression analysis compared with PCR and microarray-based techniques and improves the efficiency of characterizing miRNAs in vismodegib-treated BCC [24]. Second, we aimed to describe our surgical experience with neoadjuvant vismodegib-treated BCC, which has been discussed in the literature as a possible alternative treatment regimen in otherwise unresectable BCC [25, 26].

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**materials and methods**

The present study originated from the Dermatological Surgery Section at an academic university hospital and the Department of Plastic Surgery at a regional medical center with a focus on skin and facial surgery. The skin biopsies carried out in the present study were conducted in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki Declaration and its later amendments and comparable ethical standards and were approved by the Ethical Review Board of the Ruhr-University Bochum. Informed consent was obtained from the patient.

**subject and samples**

A 67-year-old female presented to our Department of Plastic Surgery with an initially 13 × 18 cm large, ulcerating BCC of the left forehead and scalp (Figure 1A). The patient was initially treated for a period of 6 months with an oral SHH pathway inhibitor (vismodegib 150 mg/day, Erivedge, Roche, Grenzach-Wyhlen, Germany). During this time, from a clinical and macroscopical point of view, the tumor was significantly reduced by >70% of its initial size (Figure 1B). During micrographic controlled surgery with cold steel under general anesthesia, a 4-mm biopsy was taken from the center of the tumor. An additional biopsy was taken from adjacent nonlesional epidermal skin (NLES) during defect closure with ultra-thin split-thickness skin grafting (NLES, control).

**RNA isolation and quality control**

The purity and concentrations of the total RNA samples were determined with NanoDrop ND-1000. The inclusion criterion for the RNA was an OD of A260/A280 ratio between 1.8 and 2.1.
Figure 1. (A) Ulcerating basal cell carcinoma of the left forehead and scalp (13.5 × 18.2 cm). (B) Ulcerating basal cell carcinoma of the left forehead and scalp after 6 months of vismodegib, 150 mg/day; the tumor has been significantly reduced by >70% of its initial size. (C) Final defect after seven microscopically controlled re-excisions to obtain tumor-free margins, with the final defect being 18.3 × 23.4 cm. (D) Three months after cranial bone drilling and ultra-thin split-thickness skin grafting.

**Ultra-high-throughput sequencing of the BCC miRNome**

**Sequencing library preparation.** Total RNA of each sample was used to prepare the miRNA sequencing library, which included the following steps: (i) 3' -adapter ligation, (ii) 5' -adapter ligation, (iii) cDNA synthesis, (iv) PCR amplification and (v) size selection of ~135–155 bp PCR-amplified fragments (corresponding to ~15–35 nt small RNAs). The total RNA of each sample was sequentially ligated to 3' and 5' small RNA adapters. The cDNA was synthesized and amplified using Illumina’s proprietary RT and amplification primers. Next, 135–155 bp PCR-amplified fragments were extracted and purified from the PAGE gel. Finally, the completed libraries were quantified by an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA).

**Cluster generation and sequencing on an Illumina HiSeq 2000**

The samples were diluted to a final concentration of 8 pM, and cluster generation was carried out on the Illumina cBot using the TruSeq Rapid SR cluster kit (Illumina, San Diego, USA) in accordance with the manufacturer’s instructions. The libraries were denatured as single-stranded DNA molecules, captured on Illumina flow cells, amplified in situ as clusters, and finally sequenced for 36 cycles on Illumina HiSeq as per the manufacturer’s instructions (Illumina). Sequencing was carried out on Illumina HiSeq 2000 using the TruSeq Rapid SBS kit (Illumina) according to the manufacturer’s instructions.

**Data collection and analysis**

The clean reads that passed the quality filter were processed to remove the adapter sequence as trimmed reads. Raw sequences were generated as clean reads from the Illumina HiSeq by real-time base calling and quality filtering. The clean reads were recorded in FASTQ format, including the read information, sequences and quality encoding. Next, the 3′-adapter sequence was trimmed from the clean reads. Reads with lengths shorter than 15 nt were discarded. Because the 5′-adapter sequence was also used as the sequencing primer site, this sequence was not present in the sequencing reads. The trimmed reads were recorded in FASTQ format. The trimmed reads (length ≥ 15 nt) were aligned to the human pre-miRNA in miRBase 21 using the NovoAlign software (Novocraft Technologies, Selangor, Malaysia) [27]. For miRNA alignment, the maximum mismatch was 1. Reads with counts < 2 were discarded when calculating miRNA expression. The miRNA read counts were normalized as tag counts per million miRNA alignments [28]. The miRNA read counts were used to estimate the expression level of each miRNA. The sequence counts for all miRNAs in the samples are presented in Table 1. Both samples were compared by ‘fold changes’ (FCs) of differentially expressed miRNAs. MiRNAs having FCs ≥ 2 and read count differences (RCDs) ≥ 10 were selected as differentially expressed miRNAs. The analysis outputs were filtered and ranked according to FC using Microsoft Excel’s Data/Sort and Filter functionalities (Microsoft, Redmond, USA). Novel miRNA prediction was carried out with the miRDeep algorithm as previously described [24].

**Results**

**Quality control**

The RNA OD A260/A280 ratios were 1.8 and 1.9. For quality assessment of the sequencing library, an Agilent 2100 Bioanalyzer and Agilent DNA 1000 chip kit (Agilent) were used according to the manufacturer’s protocols. All quality criteria for successful miRNA sequencing were fulfilled.

**Identification of differentially expressed miRNAs in BCC**

Pearson’s correlation coefficient R for the BCC and control samples showed a high degree of correlation (R = 0.936; Figure 2). However, we were able to describe 33 up-regulated miRNAs, with 13 miRNAs showing (FC ≥ 2; RCD ≥ 10; Table 1). The top 10 up-regulated miRNAs were hsa-miR-223-3p, hsa-miR-197-3p, hsa-miR-342-3p, hsa-miR-505-3p, hsa-miR-204-5p, hsa-miR-941, hsa-miR-145-5p, hsa-miR-301b-3p, hsa-miR-452-5p...
and hsa-miR-191-5p. The full list, including the mature seed sequence, tag count difference BCC versus control skin and FC, is given in Table 1.

novel miRNA candidates

A total of 39 novel miRNA candidates were identified, with 16 candidates showing miRDeep scores >1 (Table 2). The predicted novel miRNA, chromosome location of the predicted pre-miRNA (provisional id), miRDeep2 score, total read count, consensus precursor sequence, consensus mature sequence and estimated probability that the miRNA candidate is a true positive are given in Table 2. The top seven miRNA candidates (sorted by the miRDeep2 score) were the subject of a sequence analysis by co-author M. R. Friedländer regarding their sequence structures and likelihoods of being true positive novel miRNAs. Candidate chr1_1913 showed reads from both hairpin arms and overlaps with a small nucleolar RNA, or snoRNA, which is known to be cleaved into miRNA-like transcripts (Figure 3). Candidate chr1_1913 was therefore determined to be promising [29]. Candidate chr1_421 was determined to be borderline, although it had reads from both arms in which there were only a few reads. The remaining miRNA candidates were not promising.
After an initial excision with cold steel under general anesthesia, dermatohistopathology revealed that skip lesions were present throughout the entire tumor and showed subclinical residual tumor nests in macroscopic tumor-free skin (Figure 4). This unfortunately was the reason we had to perform seven microscopically controlled re-excisions to obtain tumor-free margins, with the final defect being larger in size (18.3 × 23.4 cm) compared with the clinical tumor size presented during the first consult, at which time neoadjuvant vismodegib therapy was initiated (Figure 1C). Reconstructive surgery was carried out by using a round bone drill to induce pin-point bleeding on the cranial bone to allow ultra-thin split-thickness skin grafting from the left thigh, which was uneventful (Figure 1D).

Figure 4. Basal cell carcinoma skip lesions stained with anti-human CD326 showing subclinical residual tumor nests in macroscopic tumor-free skin.

surgical therapy

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discussion

differentially expressed miRNAs

Regarding miRNAs, several studies have characterized differentially expressed miRNAs in BCC; however, there have been no data based on an NGS approach. Here, we described a group of up-regulated miRNAs by miRNA sequencing. When comparing the results of miRNA sequencing and previous microarray and RT-PCR miRNA profiling studies describing differentially expressed miRNAs in BCC based on FCs, we observed some similarities. Hsa-miR-301b-3p, a member of the oncogenic hsa-miR-130/301 seed family, which consists of hsa-miR-130a, hsa-miR-130b, hsa-miR301a and hsa-miR301b, has previously been shown to be up-regulated in BCC and promote invasiveness in pancreatic carcinoma cells by targeting p63 [20, 30]. All members of the oncogenic hsa-miR-130/301 seed family share one identical 5’ seed sequence; thus, similar targets for this group are expected. Hsa-miR-505-3p has also previously been shown to be up-regulated in BCC, a finding similar to those of the present analysis [20]. A variety of other cancers, such as bladder, breast, ovarian and pancreatic cancer, have shown differential expression of hsa-miR-505-3p [31–33]. Similar to BCC in the present report, hsa-miR-223-3p has likewise been shown to be up-regulated in cutaneous squamous cell carcinoma and down-regulated in mycosis fungoides, the most common form of cutaneous T-cell lymphoma [34, 35]. The V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B gene has been shown to be targeted by hsa-miR-223-3p in nasopharyngeal carcinoma cells, which reduces their growth and migration rate [36]. Hsa-miR-197-3p has been shown to be down-regulated in psoriatic skin lesions; its over-expression inhibits keratinocyte proliferation and migration by IL-22 [37]. Furthermore, the IL-22 receptor subunit IL22RA1 has been found to be targeted by hsa-miR-223-3p [38], which leads to reduced adhesion, migration and invasion of squamous cell carcinoma of head and neck cells in vitro [39, 40]. In an miRNA profiling study, hsa-miR-145-5p was found to be up-regulated and described as suitable for early detection of NMSC with plasma miRNAs [41]. Hsa-miR-301b-3p previously was shown to be up-regulated in a variety of cancers such as pancreatic, colorectal and oral cancer, and stimulates cell invasiveness in pancreatic carcinoma cells [30]. The expression of hsa-miR-452-5p has been described as altered during the healing of diabetic wounds [42]. Hsa-miR-191-5p has a pivotal effect on the senescence and proliferation of keratinocytes. It has been shown that hsa-miR-191-5p is up-regulated in senescent human epidermal keratinocytes and targets SATB1 and CDK6 3’ UTRs, thereby triggering keratinocyte senescence [43]. Silencing these hsa-miR-191-5p targets induced a G1 block in the keratinocyte cell cycle. Furthermore, it is interesting to note that on the basis of an FC ≥2, no miRNA down-regulation was observed. If this is a vismodegib-specific effect, it has not been evaluated so far; however, previous miRNA expression studies in nonvismodegib-treated BCC showed significantly down-regulated miRNAs (FC ≥2). Thus, a high degree of conformability could be observed based on this NGS miRBase21 miRNA sequencing approach, compared with RT-PCR and microarray data based on previous miRBase versions. As a limitation, however, one has to keep in mind that low-to-medium read count differences between 10 and 30 should be verified with additional analysis.

novel miRNA candidates

On the basis of the analysis of putative new miRNA sequences, two promising new miRNA candidates were analyzed. The mature sequence of a borderline candidate, chr1_421, contains a mismatch that could be a single-nucleotide polymorphism, but could also indicate that this candidate actually originates from a different genomic location. The candidate overlaps with a divergent short interspersed nuclear transposable element, which is known to give rise to miRNA-like transcripts [44]. Both candidate sequences should be further characterized in functional Dicer knockout studies to determine whether they are true new miRNA sequences.

surgical approach

From a surgical point of view, we were able to confirm the findings of previous reports that determined that BCC was dramatically reduced in size under oral vismodegib therapy. Unfortunately, the histopathologic evaluation of tumor-free margins was complicated as residual tumor nests were found beyond cleared margins, which from our point of view, drastically limits a neoadjuvant approach with oral vismodegib as seven re-excisions were necessary in the present case [25, 45]. Although this study described a single case, we think that further neoadjuvant approaches for vismodegib are limited because of impaired histopathologic evaluations. Larger studies and case series with longitudinal approaches are necessary to evaluate this important clinical issue in the future.

conclusion

The present study describes differentially expressed miRNAs in vismodegib-treated BCC tissue and suggests that further evaluations of neoadjuvant approaches with vismodegib in BCC are necessary as there is initial evidence that a higher rate of surgical excision is necessary to reach tumor-free margins.

acknowledgements

This study conforms to applicable local requirements regarding ethical and investigational committee review, informed consent and other statutes or regulations regarding the protection of the rights and welfare of human subjects participating in medical research (Ethical Review Board of the Ruhr-University Bochum, Germany).

funding

DS is supported by the Heed Ophthalmic Foundation (no grant number).
disclosure
All authors hereby disclose no commercial associations that may pose or create a conflict of interest with the information presented in this manuscript. The authors alone are responsible for the content and writing of the paper. The authors have declared no conflicts of interest.

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