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Next generation sequencing reveals microRNA isoforms in liver cirrhosis and hepatocellular carcinoma☆

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ABSTRACT

Hepatocellular carcinoma (HCC) represents the major histological subtype of liver cancer. Tumorigenic changes in hepatic cells potentially result from aberrant expression of microRNAs (miRNAs). Individual microRNA gene may give rise to miRNAs of different length, named isomiRNAs that proved to be functionally relevant. Since microRNA length heterogeneity in hepatic tissue has not been described before, we employed next-generation sequencing to comprehensively analyze microRNA transcriptome in HCC tumors ($n = 24$) and unaffected tissue adjacent to tumors ($n = 24$), including samples with ($n = 15$) and without cirrhosis ($n = 9$).

We detected 374 microRNAs expressed in liver, including miR-122-5p that constituted over 39% of the hepatic miRnome. Among the liver expressed miRs, the levels of 64 significantly differed between tumor and control samples (FDR < 0.05, fold change > 2). Top deregulated miRNAs included miR-1269a (T/N = 22.95), miR-3144-3p (T/N = 5.24), miR-183-5p (T/N = 4.63), miR-10b-5p (T/N = 3.87), miR-490-3p (T/N = 0.13), miR-199a-5p (T/N = 0.17), miR-199a-3p/miR-199b-3p (T/N = 0.19), miR-214-5p (T/N = 0.20) and miR-214-3p (T/N = 0.21). Almost all miRNA genes produced several mature molecules differing in length (isomiRNAs). The reference sequence was not the most prevalent in 38.6% and completely absent in 10.5% of isomiRNAs. Over 26.1% of miRNAs produced isoforms carrying ≥ 2 alternative seed regions, of which 35.5% constituted novel, previously unknown seeds. This fact sheds new light on the percentage of the human genome regulated by microRNAs and their variants. Among the most deregulated miRNAs, miR-199a-3p/miR-199b-3p (T/N fold change = 0.18, FDR = 0.005) was expressed in 9 isoforms with 3 different seeds, concertedly leading to upregulation of TGF-beta signaling pathway (OR = 1.99; $p = 0.004$).

In conclusion, the study reveals the comprehensive miRNome of hepatic tissue and provides new tools for investigation of microRNA-dependent pathways in cirrhotic liver and hepatocellular carcinoma.

This article is part of a Directed Issue entitled: Rare Cancers.

1. Introduction

Incidence of liver malignancies is estimated in whites at 3.8 and 1.4/100.000 in men and women, respectively, but is much higher in Asian and African populations (Jemal et al., 2011). Hepatocellular carcinoma (HCC) represents the major histological subtype, amounting to 70–85% of all hepatic cancer cases. The most important risk factors for HCC include liver cirrhosis resulting from hepatitis B or C virus infection, alcohol abuse or exposure to aflatoxin. Somatic mutations in HCC are relatively rare, and concern mainly tumor suppressors and oncogenes, such as *TP53* or *CTNNB1*, leading to activation of WNT and β-catenin pathways. Other mutations, located in *IGF2R*, *SMAD2* and *SMAD4* lead to aberrances in the TGF-β pathway, while disturbances in cell cycle control result from

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mutations in *RB1*, *P16INK4A* or *CCND1* (Bruix et al., 2004; Hussain et al., 2007; Tornesello et al., 2013). All of the abovementioned genetic mutations are important factors implicated in hepatocarcinogenesis, but cannot explain all the disturbances in gene expression observed in liver tumors. A search for epigenetic alterations resulted in identification of numerous microRNAs, whose expression is aberrant in hepatocellular carcinoma (Negrini et al., 2011).

MicroRNAs (miRNAs, miRs) are short, non-coding RNAs that bind to complementary sequences in 3′untranslated regions of protein-coding genes and regulate their expression (Bartel, 2009; Filipowicz et al., 2008). Aberrant expression of miRNAs results in aberrant expression of their target mRNAs and is implicated in development of several human cancers (Calin and Croce, 2006). A single miRNA regulates the expression of numerous genes (Lim et al., 2005), and several miRNAs might concertedly regulate the expression of a single gene, whose function is pivotal in a given tissue (Jazdzewski et al., 2011).

The recognition of mRNA by a microRNA depends on the "seed region" of a miR, comprising nucleotides 2–8 of mature molecule (Nielsen et al., 2007). Sequences of miRNAs are deposited in a public repository – miRBase (Griffiths-Jones, 2006), listing all the known, reference miRNAs. Recent studies showed that individual miRNA gene might give rise to numerous mature miRNA molecules of varying length, named isomiRs (Morin et al., 2008). IsomiRs may originate from imperfect specificity of both Drosha and Dicer cleavage of microRNA precursors, mainly due to asymmetrical structural motifs present in precursor hairpins or from trimming or extension of mature miRs (Chiang et al., 2010; Starega-Roslan et al., 2011). Sequence variations of many of the isomiRs consist in addition or deletion of nucleotides at their 5′end when compared to the reference miRNA, resulting in a change of the miR's "seed region" and, in consequence, leading to recognition and regulation of distinct sets of target genes. IsomiRs are tissue-specific and functional partners of their reference miRNAs (Cloonan et al., 2012; Humphreys et al., 2012; Kozlowska et al., 2013; Llorens et al., 2013; Wojcicka et al., 2014). Most studies on cancer-related microRNA aberrances were based on the analysis of expression of canonical, reference miRNAs, as the analysis of isomiRs requires the use of more robust technologies, such as next-generation sequencing, and laborious analysis of the obtained data. The role of isomiRs in pathogenesis of cancers has been so far shown in a murine model of leukemia (Kuchenbauer et al., 2008) as well as in human melanoma (Kozubek et al., 2013) and papillary thyroid carcinoma (Swierniak et al., 2013).

MicroRNAs seem to be vitally implicated in liver carcinogenesis. MiR-122-5p is the most abundant microRNA in hepatic tissue (Tsai et al., 2009). Its decreased levels, observed in HCC, result in increased expression of cyclin G1 and a consequent deregulation of p53 – related pathways (Gramantieri et al., 2007). On the other hand, miR-122 binds to the 5′ untranslated region of hepatitis C virus, enhancing its replication (Wang et al., 2012) but, surprisingly, chronic HCV infection leads to downregulation of miR-122 in hepatic tissue (Choi et al., 2013; Li et al., 2013). Other miRs, often downregulated in liver pathologies, include let-7 family, miR-26a/b, miR-138 or miR-199a/b that target genes involved in cell-cycle control and proliferation. Liver cirrhosis and HCC risk are also associated with polymorphisms in microRNA sequences, such as rs11614913 (C→T) in a precursor of miR-196a2 (Li et al., 2010; Qi et al., 2010), and rs2910164 in miR-146a (Xu et al., 2008). This effect was explained by the fact that presence of the rare alleles severely lowers synthesis of both miRs expressed from a single precursor and leads to altered gene recognition by the variant miRs (Hoffman et al., 2009; Hu et al., 2008; Jazdzewski and de la Chapelle, 2009; Jazdzewski et al., 2008, 2009). Since the target genes for miR-196a include mediators of apoptosis and Hox genes, its aberrant expression can lead to severe changes in cellular pathways and initiate the process of tumorigenesis (Hornstein et al., 2005; Luthra et al., 2008).

Even though next-generation sequencing was implemented to identify novel miRNAs in HCC-derived cell lines (Law et al., 2013), no reports on the liver-specific isomiRs have been published so far. Thus, in this study, we employed next-generation sequencing to identify all the microRNA isoforms that are expressed in liver tissue and whose aberrances can potentially underlie initiation and progression of carcinogenesis.

2. Materials and methods

2.1. Liver tissue samples

Specimens from HCC tumors (HCC-T, $n = 24$) and unaffected tissue adjacent to, but not infiltrated by tumor from the same patient (HCC-N, $n = 24$) were collected at the Medical University of Warsaw, Poland. The clinical characteristics of patients are summarized in Supplementary Table 1. After obtaining the approval of the Institutional Review Board and patient consent, fresh tissue samples were taken during surgical resection, snap-frozen on dry ice and stored at −80 °C. Total RNA was extracted with TRIzol solution (Invitrogen), and the integrity of RNA was assessed using Agilent BioAnalyzer 2100 (Agilent).

Supplementary Table 1 associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.05.020.

2.2. Next-generation sequencing

Small RNA fraction (with length not exceeding 50 nt) was subjected to hybridization and ligation with Adaptor Mix (New England Biolabs). Subsequently, RNAs were reverse transcribed and sequenced using whole-transcriptome sequencing on the Illumina HiSeq2000 platform.

2.3. Data extraction and analysis

To detect all human isomiRs, a library of reference sequences was prepared by identifying the sequences of mature miRNAs, together with 5 flanking nucleotides, within the hairpins deposited in miRBase, version 19 (Kozomara and Griffiths-Jones, 2011). The Illumina HiSeq platform requires a minimum read length of 50 nucleotides, thus all the small RNAs were extended using specific adapters that were annealed to their 3′ends during library preparation. The removal of adapters was performed in silico on the raw, 50-nucleotide-long Illumina sequence reads using cutadapt software (Martin, 2011). The resulting sequences of the length of 15–28 nucleotides were subjected to further analysis as potential miRNAs. The sequences were mapped on the prepared reference library using Bowtie v 0.12.7 (Langmead et al., 2009), with the requirement of perfect matching. The numbers of mapped reads were subsequently calculated for each miRNA and provided in two ways as (1) a number of each of unique reads mapped to each reference sequence, and (2) a number of all reads mapped to each reference sequence. Data obtained for each sample was normalized using the RPM (reads per million) normalization according to the formula: $\mathrm{RPM} = (N_{\mathrm{ref}}/N_{\mathrm{all}}) \times 10^6$, where: N_{ref} – number of reads mapped to the miRNA reference, N_{all} – total number of reads mapped in the sample.

2.4. Statistical analysis

Selection of miRNAs and isomiRs deregulated between the analyzed groups was performed using Welch *t*-test – paired for comparison between HCC-T and HCC-N samples, and unpaired for