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Expression signatures of early-stage and advanced medaka melanomas

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ABSTRACT

Melanoma is one of the most aggressive tumors with a very low survival rate once metastasized. The incidence of newly detected cases increases every year suggesting the necessity of development and application of innovative treatment strategies. Human melanoma develops from melanocytes localized in the epidermis of the skin to malignant tumors because of deregulated effectors influencing several molecular pathways. Despite many advances in describing the molecular changes accompanying melanoma formation, many critical and clinically relevant molecular features of the transformed pigment cells and the underlying mechanisms are largely unknown. To contribute to a better understanding of the molecular processes of melanoma formation, we use a transgenic medaka melanoma model that is well suited for the investigation of melanoma tumor development because fish and human melanocytes are both localized in the epidermis. The purpose of our study was to gain insights into melanoma development from the first steps of tumor formation up to melanoma progression and to identify gene expression patterns that will be useful for monitoring treatment effects in drug screening approaches. Comparing transcriptomes from juvenile fish at the tumor initiating stage with nevi and advanced melanoma of adults, we identified stage specific expression signatures and pathways that are characteristic for the development of medaka melanoma, and are also found in human malignancies.

1. Introduction

Malignant melanoma is the most aggressive form of skin cancer with a rapidly rising incidence worldwide (Siegel et al., 2014). Melanoma rates have doubled in the past 30 years and an estimated 87,110 new melanoma cases will be diagnosed in 2017 in the United States (https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2017/cancer-facts-and-figures-2017.pdf). Primary melanoma tends to metastasize to many different parts of the body and at that stage current therapeutic strategies frequently fail. New treatment options were established and recently approved increasing the one-year progression free survival up to 50% (CheckMate 067 study, Larkin et al., 2017). Among these are immunotherapies (CTLA4- and PD1-inhibitor) and targeted therapies (BRAF- and MEK-inhibitors) with good response rates. However, side effects and drug resistant tumors may compromise treatment success (Chapman et al., 2011; Eggermont et al., 2016; Luke et al., 2017). In addition, there remain a large number of patients that do not benefit at all from these new treatments, e.g. patients with wild-type BRAF status. Therefore, the improvement of existing and the development of new therapies are important health care priorities. Comprehensive understanding of the genetic profile of melanoma primary tumor development and progression is a precondition for the development of new therapeutic options and strategies. Predisposing factors for malignant melanoma include exposure to ultraviolet radiation as well as pigmentation characteristics such as fair skin, a high number of nevi (Markovic et al., 2007) as well as genetic risk factors. Aberrations in different genes such as *CDKN2a*, *P53* or *BRAF* are associated with

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familial melanoma (Tsao et al., 2012). Genome sequencing projects of human metastatic melanomas have uncovered a large number of somatic alterations. Among these are the already known oncogenes *BRAF* (in about half of all melanomas) and *NRAS*, but also new candidates, like *NF1* and *PREX2* (Berger et al., 2012; Cancer Genome Atlas, N., 2015). The identification of such new candidate genes is very important for a better understanding of the contribution of genetic and genomic alterations to melanoma formation. Insights into melanoma tumor biology were also provided by a large number of genetic and molecular studies; however, they are only retrospective (Chin et al., 2006; Hodis et al., 2012; Sturm et al., 1994).

Cancer is a systemic disease. Therefore, the use of animal models with high anatomical and physiological similarities to humans is extremely important for the investigation of developmental and environmental factors that drive melanoma formation. Small aquarium fish, such as zebrafish or medaka are well suited for biomedical research because of several features and are well-accepted models to study melanoma development (Patton et al., 2010). Both have high fecundity, short generation time, genetic tractability and they are easy to breed in large numbers (Lieschke and Currie, 2007; Wittbrodt et al., 2002). We use pigment cell tumor-developing transgenic medaka as model for studying melanoma (Schartl et al., 2010). Melanocytes in fish, like in humans, are localized in the basal layer of the epidermis in the skin. In contrast, mouse melanocytes are located in the hair follicles and in the dermis (Gola et al., 2012; Kelsh, 2004). This anatomical conformity together with a high degree of molecular genetic conservation between medaka and human pigment cell cancers (Patton et al., 2010; Schartl et al., 2012) make medaka a highly suitable in-vivo study system.

Development of small aquarium fish models of melanoma has been proposed to produce superior experimental systems for high throughput first line drug screens (van Rooijen et al., 2017). Successful screening experiments in zebrafish have been conducted, however, these utilized established screening technologies by exposing embryos to chemicals and monitoring drug effects on normal melanocytes (Colanesi et al., 2012; White et al., 2011). So far – with a single exception (Matsuzaki et al., 2013) – efficient systems to monitor the effect of drugs that act on developing melanoma in free swimming larvae or adult animals have not been devised and drug screening protocols on larvae and adults are lacking, partly due to the difficulty of monitoring phenotypic changes of a fully developed melanoma and the long-term that such changes require to become visible. Reasoning that drug effects would be first visible at the level of gene expression, we are working toward developing such gene expression patterns that hallmark melanoma. Our ability to find gene expression readouts of melanoma development depends critically on the knowledge of transcriptional changes (i.e. expression signatures) in tumors at different stages of development.

In this study, we used a pigment cell tumor developing medaka model for investigating gene expression profiles of early-stage in juveniles (3–5 weeks) and advanced melanoma in adults (6–9 months). These fish are transgenic for the *xmrk* oncogene from *Xiphophorus maculatus* under the pigment-specific promoter of the medaka *mitfa* gene. They develop aggressive melanomas, which are highly invasive into the internal organs of the body, and form large nodular tumors in the skin and at extracutanous sites (Schartl et al., 2010). The purpose of this study was to identify transcriptional profiles of juvenile fish at the initial stage of melanoma formation in comparison to nevi and advanced melanoma from adult medaka. We found a clear downregulation of critical genes from the innate and adaptive immune system in the early stage of melanoma development while an upregulation of adaptive immune response genes were detected in melanoma tumors from adults. Differentially regulated pigment cell and pigmentation specific genes were identified in both early and advanced stage melanomas while other gene expression patterns constitute specific transcriptional signatures of the respective tumor stage.

2. Material and methods

2.1. Fish maintenance

Transgenic *Oryzias latipes* (medaka) of the Carbio Tg(*mitf*:*xmrk*) strain (Schartl et al., 2010) were used as model to investigate melanoma-specific transcriptional signatures. Fish of this strain carry the cDNA from the melanoma-inducing oncogene *xmrk* of *Xiphophorus maculatus*. The expression of *xmrk* under the pigment cell specific medaka *mitfa* promotor results in formation of pigment cell tumors with up to 100% penetrance. All fish were kept under standard conditions in the aquarium facility of the Biocenter at the University of Wuerzburg in compliance with local animal welfare laws, guidelines and policies and under the authorization (55.2 – 2531.01 – 40/14) of the Veterinary Office of the District Government of Lower Franconia, Germany, in accordance with the German Animal Protection Law.

2.2. RNA-sequencing (RNA-seq)

Total RNA was isolated from whole body of 10 individual transgenic and 10 individual non-transgenic (wild-type) fish using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The age of the fish ranged from three to five weeks at a uniform total length of about 1 cm. To increase the sample size and for cross-validation we added to the dataset produced in this study the primary data from an earlier study (Schartl et al., 2015) with four wildtype and four melanoma juveniles of the same age and size, and reanalyzed them in comparison with our new data. RNA from adults (6–9 months old) was extracted from pooled fins of 11 non-transgenic medaka (strain Carbio), 2 pools (n = 5 and 10) of nevi from two independent dissections from transgenic fish (Tg(*mitf*::*xmrk*); Tg (*mitf*::*xmrk*), *p53* −/−) and three individual exophytic melanoma (Tg (*mitf*::*xmrk*)) using commercially available systems (RNeasy Kit, Qiagen, Hilden, Germany). Quality of RNA was assessed by measuring the RNA Integrity Number (RIN) using an Agilent 2100 Electrophoresis Bioanalyzer Instrument G2939A (Agilent Technologies, Böblingen, Germany). RNA samples with RIN > 8 were used for sequencing. High-throughput paired-end RNA sequencing libraries were constructed following the standard Illumina mRNA library preparation protocol (www.illumina.com; Illumina Inc., San Diego, CA, U.S.A.).

2.3. RNA-seq validation by quantitative real-time PCR (qRT-PCR)

For confirmation of RNA-seq data, selected genes were analyzed by qRT-PCR analysis. Total RNA from three to five weeks old individual transgenic and wild-type medakas (n = 4–10 in each group) was extracted from whole bodies using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA from adults (6–9 months old) was isolated from one pool (n = 9) of nevi from transgenic medakas (Tg(*mitf*::*xmrk*)) and 7–8 individual exophytic melanoma (Tg(*mitf*::*xmrk*)) using TRIzol Reagent (Thermo Fisher Scientific, Waltham, USA) according to the supplier's recommendation. After DNase treatment, total RNA (1–2 µg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA) and random hexamer primers, according to the manufacturer's instructions. For real-time qRT-PCR, cDNA from 25 ng of total RNA was analyzed in triplicates using SYBR Green reagent. Primer sequences are listed in Table S1. Amplification was monitored using a Mastercycler ep realplex^2 (Eppendorf, Hamburg, Germany). For quantification, expression of each gene was normalized to the housekeeping gene *ef1a1* (elongation factor 1 alpha 1) using the delta Ct method (Simpson et al., 2000). Differences in mRNA expression levels were tested for significance using Wilcoxon–Mann–Whitney *U* test or Student's *t*-test depending on the sample size and distribution. Normally distributed data with a sample size larger than 9 were analyzed by Student's *t*-test and data with a samples size smaller than 9 or