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Original Article

Loss of photoreceptorness and gain of genomic alterations in retinoblastoma reveal tumor progression

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ABSTRACT

Background: Retinoblastoma is a pediatric eye cancer associated with *RB1* loss or *MYCN* amplification ($RB1^{+/+}MYCN^{A}$). There are controversies concerning the existence of molecular subtypes within $RB1^{-/-}$ retinoblastoma. To test whether these molecular subtypes exist, we performed molecular profiling.
Methods: Genome-wide mRNA expression profiling was performed on 76 primary human retinoblastomas. Expression profiling was complemented by genome-wide DNA profiling and clinical, histopathological, and ex vivo drug sensitivity data.
Findings: RNA and DNA profiling identified major variability between retinoblastomas. While gene expression differences between $RB1^{+/+}MYCN^{A}$ and $RB1^{-/-}$ tumors seemed more dichotomous, differences within the $RB1^{-/-}$ tumors were gradual. Tumors with high expression of a photoreceptor gene signature were highly differentiated, smaller in volume and diagnosed at younger age compared with tumors with low photoreceptor signature expression. Tumors with lower photoreceptor expression showed increased expression of genes involved in M-phase and mRNA and ribosome synthesis and increased frequencies of somatic copy number alterations.
Interpretation: Molecular, clinical and histopathological differences between $RB1^{-/-}$ tumors are best explained by tumor progression, reflected by a gradual loss of differentiation and photoreceptor expression signature. Since copy number alterations were more frequent in tumors with less photoreceptorness, genomic alterations might be drivers of tumor progression.
Research in context: Retinoblastoma is an ocular childhood cancer commonly caused by mutations in the *RB1* gene. In order to determine optimal treatment, tumor subtyping is considered critically important. However, except for very rare retinoblastomas without an *RB1* mutation, there are controversies as to whether subtypes of retinoblastoma do exist. Our study shows that retinoblastomas are highly diverse but rather than reflecting distinct tumor types with a different etiology, our data suggests that this diversity is a result of tumor progression driven by cumulative genetic alterations. Therefore, retinoblastomas should not be categorized in distinct subtypes, but be described according to their stage of progression.

1. Introduction

Retinoblastoma is a childhood cancer of the retina, usually caused by bi-allelic inactivation of the *RB1* tumor suppressor gene. In 40% of the cases, patients have a hereditary predisposition due to the presence of a germ line mutation in *RB1*. Only one somatic inactivation of *RB1* is required in hereditary patients to develop retinoblastoma and therefore they are often affected bilateral. While the non-hereditary form of

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retinoblastoma is usually caused by somatic inactivation of both *RB1* alleles, a subtype of retinoblastoma was recently described which lacks mutations in *RB1* but displays high level amplification of the oncogene *MYCN* (Rushlow et al., 2013). Besides the initiating hit (*RB1* mutation or *MYCN* amplification), additional DNA mutations are likely required for retinoblastoma to develop (Dimaras et al., 2008). Common chromosomal alterations observed in retinoblastomas are gains of chromosomal regions 1q, 2p and 6p, and losses at chromosome 16q (Thériault et al., 2014). We and others have previously described differences in the level of chromosomal instability between retinoblastomas, depending on age of the patient, heritability and laterality (Herzog et al., 2001; Lillington et al., 2003; Mol et al., 2014; Sampieri et al., 2009; van der Wal et al., 2003; Zielinski et al., 2005).

In addition to copy number analyses, several gene expression studies on retinoblastoma have been published (Chakraborty et al., 2007; Ganguly and Shields, 2010; Kapatai et al., 2013; McEvoy et al., 2011). It has been suggested that retinoblastomas have similar expression profiles and express genes involved in multiple differentiation programs (McEvoy et al., 2011). However, in another recent study (Kapatai et al., 2013), two different subtypes of retinoblastoma were identified based on gene expression profiling. One group expressed genes associated with a range of different retinal cell types, suggesting a progenitor cell of origin, while the second group showed high expression of cone photoreceptor associated genes, suggesting derivation from a cone photoreceptor cell precursor. So, while it is well-established that there are 2 genomic subtypes ($RB1^{-/-}$ and $RB1^{+/+}MYCN^{A}$) there is controversy surrounding gene expression subtypes, in particular within the $RB1^{-/-}$ tumor subtype.

To address this issue, we have performed transcriptome-wide expression profiling of a large, diverse and randomly selected set of retinoblastomas. Subsequently, expression profiles were associated with copy number profiles, clinical characteristics and ex vivo drug sensitivity data.

2. Material and Methods

2.1. Patient Samples

Retinoblastoma samples from a consecutive patient series were collected after primary enucleation (without receiving previous treatment) at the VU University Medical Center (Amsterdam, The Netherlands), which is the national retinoblastoma referral center in the Netherlands. Incisions were made in the enucleated eyes and tumor samples were taken and immediately snap frozen in liquid nitrogen and stored at −80 °C. In some cases, an additional tumor sample was used for culturing, as described previously (Schouten-van Meeteren et al., 2001). Histopathology was determined at initial pathological diagnosis by a retinoblastoma-experienced pathologist and independently by an ophthalmologist and pediatric oncologist. Tumor location was determined on funduscopy results and/or fundus photos by an ophthalmologist. The disease was staged according to the two most common classification systems for retinoblastoma, the Reese–Ellsworth Classification (Reese and Ellsworth, 1963) and the International Intra-ocular Retinoblastoma Classification (ABC-classification) (Linn Murphree, 2005). This prospective study was conducted in accordance with recommendations of the local ethics committee, with waiver of informed consent (IRB00002991 reference 2014.360).

2.2. DNA Extraction and Copy Number Profiling

Genomic DNA from frozen tumor retinoblastoma specimens was isolated with the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). DNA quality was analyzed for high molecular bands >20 kb by agarose gel electrophoresis. DNA concentration and OD 260/280 ratio were determined with the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). DNA yields and quality were within the same range for all samples. Microarray-based DNA genotyping experiments were performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the HumanOmni1-Quad BeadChip (Illumina Inc., San Diego, U.S.A.), as described previously (Mol et al., 2014).

2.3. RNA Extraction and Expression Profiling

Frozen tumor samples were homogenized in a TRIzol reagent (Invitrogen, Carlsbad, California, U.S.A.) with a rotor–stator homogenizer, and RNA was extracted following the manufacturer's instructions. TRIzol extracted RNA was treated with rDNase (Macherey-Nagel, Düren, Germany) to digest any contaminating DNA and subsequently purified with the NucleoSpin RNA Clean-up XS kit (Macherey-Nagel). Three samples (VUMC-Rb-76, VUMC-Rb-81, and VUMC-Rb-82) were extracted with the AllPrep RNA/DNA/Protein Mini Kit (Qiagen, Venlo, The Netherlands), following the manufacturer's instructions. Quality control, RNA labeling, hybridization and data extraction were performed at ServiceXS B.V. RNA concentration was measured using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). The RNA quality and integrity were determined using Lab-on-chip analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.) and on the Shimadzu MultiNA RNA analysis chips (Shimadzu Corporation, Kyoto, Japan). Only RNA samples that passed the quality criteria of an OD 260/280 ratio of ≥1.8 and an RNA Integrity number (RIN) of ≥7 were processed for expression profiling. Biotinylated cRNA was prepared using the Affymetrix 3′ IVT Express Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's specifications with an input of 100 ng of total RNA. The quality of the cRNA was assessed using the Shimadzu MultiNA in order to confirm if the average fragment size was according to requirements of Affymetrix. Per sample, 7.5 μg cRNA of the obtained biotinylated cRNA samples was fragmented and hybridized in a final concentration of 0.0375 μg/μL on the Affymetrix [HT HG U133 + PM96] (Affymetrix, Santa Clara, California, U.S.A.). After an automated process of washing and staining by the GeneTitan machine (Affymetrix) using the Affymetrix HWS Kit for GeneTitan (part nr. 901530), absolute values of expression were calculated from the scanned array using the Affymetrix Command Console v3.2 software. Micro-array data is available at Gene expression omnibus (GSE59983).

2.4. Micro-array Data Analysis

Absolute expression values were normalized with robust multichip array (RMA) normalization implemented by affy Bioconductor (Gautier et al., 2004) package and log2-transformed. For each official HGNC symbol targeted by multiple probes, only the probe closest to the 3′-prime end was used for further analysis. Agglomerative hierarchical (Ward, complete-linkage, average-linkage and McQuitty) clustering was performed on pairwise inverse absolute Pearson correlations. Differential expression testing was performed by generalized linear modeling of indicated (co-)variates on normalized log2-transformed expression values implemented by limma Bioconductor package (Smith, 2005). Obtained p-values were corrected for multiple hypothesis testing by Benjamini & Hochberg false discovery rate (FDR) adjustments (Benjamini et al., 1995). Obtained FDR-adjusted two-sided p-values < 0.05 were considered significant.

Copy number estimates and allelic intensity ratios exported by Illumina Beadstudio were normalized with tQN-procedure (Staaf et al., 2008). Subsequently, log2-R-ratios (LRR) were calculated from normalized copy number estimates of the tumors and matched blood samples. For tumors with no matching blood sample, sex-matched pooled baselines from all blood samples were used to calculate log2-R ratios. CGHcall (van de Wiel et al., 2007) and CGHregions (Van de Wiel and Van Wieringen, 2007) implementing the DNAcopy segmentation algorithm were used to create a reduced segment matrix containing five copy number levels. All parameters used for copy number segmentations (performed by "segmentData" function) used were defaults except the definition of small segments (clen = 25) and the amount of