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Targeted serum miRNA (TSmiR) test for diagnosis and follow-up of (testicular) germ cell cancer patients: A proof of principle

Ad J.M. Gillis[a,1], *Martin A. Rijlaarsdam*[a,1], *Ronak Eini*[a], *Lambert C.J. Dorssers*[a], *Katharina Biermann*[a], *Matthew J. Murray*[b], *James C. Nicholson*[b], *Nicholas Coleman*[c], *Klaus-Peter Dieckmann*[d], *Gazanfer Belge*[e], *Jörn Bullerdiek*[e], *Tom Xu*[f], *Nathalie Bernard*[f], *Leendert H.J. Looijenga*[a,*]

[a]*Department of Pathology, Erasmus MC – University Medical Center Rotterdam, Building Be, Room 432, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands*
[b]*Department of Paediatric Haematology and Oncology, Addenbrooke's Hospital, University of Cambridge, Cambridge, United Kingdom*
[c]*Department of Pathology, University of Cambridge, Cambridge, United Kingdom*
[d]*Department of Urology, Albertinen-Krankenhaus, Hamburg, Germany*
[e]*Department of Human Genetics, University of Bremen, Germany*
[f]*Applied Biosystems, Europe and USA, Part of Life Technologies Group, Foster City, United States*

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ABSTRACT

Germ cell cancers (GCC) are the most frequent malignancy in young Caucasian males. GCC can consist of seminomas (SE) and non-seminomas (malignant NS: embryonal carcinoma (EC), yolk sac tumor (YS), choriocarcinoma (CH) and teratoma (TE)). Current serum-markers used for diagnosis and follow-up (AFP, hCG) are predominantly related to YS and CH and marker positivity can vary during disease. Therefore, stable markers consistently identifying more GCC components, specifically the stem cell components SE and EC, are of interest. Expression of the embryonic stem cell miR-371-3 and miR-302/367 clusters in SE/EC/YS suggest possible application of these micro-RNAs as GCC tumor-markers. The TSmiR protocol constitutes a complete, quality-controlled pipeline for the detection of miRs in serum, based on magnetic bead-based purification and qPCR quantification. As a proof of principle, TSmiR was applied to five independent serum sample series including 80 GCCs, 47 controls, 11 matched pre/post orchidectomy samples and 12 no-GCC testicular masses. GCC serum samples showed a consistent, significant ($p < 0.0064$) increase of miR-371/372/373/367 levels. Analogous, serum levels returned to baseline after orchidectomy (stage-I disease). Moreover, there was a trend toward higher miR levels in patients with metastasis. These results imply suitability for diagnosis and follow-up. TSmiR showed an overall sensitivity of 98%, clearly outperforming the traditional serum markers AFP/hCG (36%/57%, $\text{sensitivity}_{\text{AFP}} = 3\%/45\%$; $\text{sensitivity}_{\text{hCG}} = 62\%/66\%$, SE/NS). TSmiR misclassified one tumor as a control. Serum AFP/hCG and TSmiR combined identified all T samples correctly. In

* *Corresponding author*. Tel.: +31 10 7044329; fax: +31 10 7044365.
E-mail address: l.looijenga@erasmusmc.nl (L.H.J. Looijenga).
[1] Both authors contributed equally to the work.

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conclusion, TSmiR constitutes a highly sensitive and reproducible serum test for GCC patients, suitable to be prospectively tested for diagnostic and follow-up purposes.

1. Introduction

Human germ cell tumors are a heterogenous group of neoplasms, derived from the germ cell lineage and originating at various stages of development with different cells of origin and pathogenesis (Oosterhuis and Looijenga, 2005). The proposed classification system has been adopted by the World Health Organization (WHO) (Woodward et al., 2004), and specialized pathologists (Reuter, 2005; Ye and Ulbright, 2012). Malignant germ cell tumors, referred to as germ cell cancer (GCC), include seminoma (SE) and nonseminoma (NS) and are predominantly found in adolescents and young adults, although also diagnosed in neonates and infants (Bray et al., 2006). It is the most frequent malignancy in young Caucasian males and incidence is increasing. GCC originate from a pluripotent embryonic germ cell (primordial germ cell/gonocyte) blocked in its maturation. This is referred to as carcinoma *in situ* (CIS) (Skakkebæk, 1972) or intratubular germ cell neoplasia unclassified (IGCNU) (Woodward et al., 2004) in the context of testicular GCC of adolescents and adults. Their pluripotency is reflected in the capacity to form the germ cell lineage (CIS/IGCNU, SE, *de novo* germ cells (Honecker et al., 2006)), embryonic stem cell components (embryonal carcinoma, EC) and all differentiation lineages (teratoma (TE), yolk sac tumor (YS) and choriocarcinoma (CH)) as found in adolescents and adults. In neonates and infants, TE and YS are distinguished, in which YS is the malignant component. Pluripotent GCC (including their precursor lesions) exhibited expression of various embryonic pluripotency markers of significant diagnostic value (OCT3/4 (POU5F1) (Looijenga et al., 2003; Rijlaarsdam et al., 2011), NANOG (Hart et al., 2005), SOX2/SOX17 (Looijenga, 2009) and LIN28 (Gillis et al., 2011; Looijenga, 2008)). In addition, CIS/SE/EC/YS components, diagnosed at pediatric and adult age, express a specific set of embryonic stem cell related microRNAs (miRs), including the miR-371-372-373 (miR-371-3) and miR302a,b,c,d/367 (miR-302/367) clusters (Gillis et al., 2007; Novotny et al., 2012; Palmer et al., 2010; Voorhoeve et al., 2006).

Depending on tumor stage and histology, pediatric and adult GCC (SE/EC/YS/CH) are effectively treatable by surgery, possibly followed by either irradiation and/or chemotherapy (Horwich et al., 2006). However, significant long term effects of the systemic treatment protocols have become evident (Haugnes et al., 2010; Van Leeuwen et al., 1993). Therefore, early and accurate diagnosis as well as detailed follow-up of GCC patients is of crucial relevance for optimal treatment, preventing possible under- or overtreatment. In clinical management of GCC, evaluation of a set of serum markers is informative for diagnosis, risk assessment and follow-up. Alpha Feto-Protein (AFP) is predominantly informative for YS and sporadically positive in EC and human Chorionic Gonadotrophine (hCG) is predominantly informative for CH with sporadic positivity in SE/EC (Horwich et al., 2006). LDH-1 has also been reported as a (less informative) serum marker (von Eyben et al., 2000). So far, no consistent markers for the stem cell components SE and EC are available, which limits the use of serum markers for diagnosis/follow-up in a large proportion of GCC patients.

Recently, three studies reported that a higher expression of members of the embryonic miR clusters miR-371-3 and miR-302/367 can be detected in serum of GCC patients (adult and pediatric) as compared to controls (Belge et al., 2012; Murray and Coleman, 2012; Murray et al., 2011). One study showed a decline to normal levels after surgical removal of stage I GCC (Belge et al., 2012). These findings could be a significant step forwards in clinical management (diagnosis and follow-up) of GCC patients, especially for the high number of "marker-negative" cases, i.e., those without elevated AFP or hCG serum levels.

The TSmiR protocol described in the manuscript constitutes a complete, quality-controlled pipeline for the detection of miRs in serum, based on magnetic bead-based purification and qPCR quantification. Using five independent sample series we show that TSmiR shows high sensitivity and specificity (GCC vs control). Also, the effect of metastasis/surgical removal of the tumor on miR levels, is investigated. This proof of concept indicates TSmiR as a potential additional tool for diagnosis & follow-up of GCC.

2. Materials and methods

2.1. Patient and control serum samples

Detailed information on the composition of the various independent sample series (R_V/R_L/UK/D/no-GCC) is presented in Figure 1A. Samples in the R_V/R_L/no-GCC series were selected to be distributed over different clinical stages and histological subtypes. R_V/R_L samples were extracted at the Department of Pathology, Erasmus MC, Rotterdam, the Netherlands. Use of tissue samples for scientific reasons was approved by an institutional review board (MEC 02.981 and CCR2041). Samples were used according to the "Code for Proper Secondary Use of Human Tissue in The Netherlands" developed by the Dutch Federation of Medical Scientific Societies (FMWV (Version 2002, update 2011). The UK and *D* samples were sent frozen to Rotterdam, handled similarly, and used in accordance to regulations set by the local institutional review boards.

2.2. miR analysis of primary GCC and normal testis

Small RNA was prepared from 47 primary, independent GCC and three normal testis samples as described in (Gillis et al., 2007). Expression of 156 miRs was analyzed, quantified and normalized according to (Mestdagh et al., 2009).