Experimental and Molecular Pathology 101 (2016) 187–196

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Investigating intra-tumor heterogeneity and expression gradients of miR-21, miR-92a and miR-200c and their potential of predicting lymph node metastases in early colorectal cancer

Rikke Karlin Jepsen *, Guy Wayne Novotny, Louise Laurberg Klarskov, Ib Jarle Christensen, Estrid Høgdall, Lene Buhl Riis

Department of Pathology and Molecular Unit, University of Copenhagen, Herlev and Gentofte Hospital, Herlev Ringvej 75, DK-2730 Herlev, Denmark

ARTICLE INFO

Article history:
Received 18 May 2016
and in revised form 16 August 2016
Accepted 19 August 2016
Available online 23 August 2016

Keywords:
microRNA-21
microRNA-200c
microRNA-92a
Early colorectal cancer
Metastases
Expression gradients

ABSTRACT

Introduction: miR-21, miR-92a and miR-200c are regulators of pathways involved in migration, intravasation and metastasis, and their tumor expression levels have been proposed as potential prognostic markers in colorectal cancer (CRC). In two parallel cohorts we examine intra-tumor expression levels in early stage CRC tissue in order to determine intra-tumor heterogeneity, potential systematic intra-tumor expression gradients of the miRNAs and to investigate the association to metastatic disease in early stage CRC.

Material and methods: Two parallel studies on archived formalin-fixed paraffin-embedded (FFPE) CRC tissue. Intra-tumor and inter-patient variances were analyzed in 9 early metastatic CRCs by measuring expression levels by qRT-PCR on isolated tissue samples from luminal, central and invasive border zones. Associations between miRNA expression levels and early metastasizing tumors was investigated in FFPE tissue from invasive border and central tumor zones from 47 early metastatic CRCs matched with 47 non-metastatic CRCs. Intra-tumor expression gradients were analyzed on both cohorts.

Results: Mean intra-tumor coefficient of variation in the heterogeneity cohort was 38.5% (range: 33.1–49.0%) only slightly less than variation between patients (45.1%, range 37.0–49.5%). We demonstrated systematic expression gradients between tumor zones equal to a 3.23 ($p = 0.003$) and 1.36 ($p = 0.014$) fold lower expression in invasive areas for miR-200c, 1.52 ($p < 0.001$) and 1.27 ($p = 0.021$) fold lower expression in invasive areas for miR-92a. For miR-21 we found a 1.75 ($p < 0.001$) and 1.21 ($p = 0.064$) fold higher expression in invasive areas compared to luminal and central zones, respectively. No significant difference in expression levels between metastatic and non-metastatic tumors was demonstrated, nor a difference in intra-tumor gradients between metastatic and non-metastatic tumors.

Conclusion: This study provides evidence for moderate intra-tumor *and* inter-patient heterogeneities of three well-described potential prognostic markers in CRC. We demonstrate intra-tumor expression gradients indicating a differentiated expression of the target miRNAs between functional tumor zones, but the potential role as markers of early metastatic disease is still not fully clarified.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide with >1.3 million patients diagnosed every year, and constitutes a major cause of morbidity and death (Ferlay et al., 2013). Prognosis is highly linked to stage at diagnosis, highlighting a need for early detection and improved individualized treatment strategies. Five year overall survival for 4317 Danish CRC patients who underwent surgery with curative intent was 75.5% for patients with localized disease (UICC stages I and II), 61.3% for patients with regional nodal spread and only 12.9% for patients with distant metastases (Danish Colorectal Cancer Group, 2014).

National screening programs are initiated with the overall goal to increase the proportion of early detected CRCs and initiation of early treatment and improvement of CRC survival. Furthermore the removal of precancerous lesions with varying degrees of neoplasia is expected to decrease the incidence of CRC (Gill et al., 2012; Friedrich et al., 2015). Identification of CRC at an early stage leads to a new challenge with regard to assessment of metastatic potential, planning of follow-up and determination of individualized treatment of patients with early CRC where subsequent surgical treatment might be indicated. In this risk stratification, classifying high-risk subgroups of patients, to avoid over- or under treatment and avoid progression of disease, is of fundamental importance.

* Corresponding author at: Department of Pathology and Molecular Unit, University of Copenhagen, Herlev and Gentofte Hospital, Herlev Ringvej 75, DK-2730 Herlev, Denmark.
E-mail address: rikke.karlin.jepsen.01@regionh.dk (R.K. Jepsen).

http://dx.doi.org/10.1016/j.yexmp.2016.08.003
0014-4800/

Management of early CRCs varies from endoscopic local excision to radical colorectal resection, the prior being sufficient in micro radically removed adenocarcinomas in polyps, pT1, in the absence of lymph node metastases (LNM). Several histopathological features such as poor differentiation, submucosal lymphovascular invasion (SLI), submucosal venous invasion (SVI), depth of invasion and tumor budding are associated with increased risk of LNM (Beaton et al., 2013; Bosch et al., 2013) and current guidelines for the pathological assessment of adenocarcinomas in polyps are based on the above high-risk features (European Colorectal Cancer Screening Guidelines Working Group et al., 2013; Danish Colorectal Cancer Group, 2010). Histopathological risk assessment may be difficult and dependent on the samples showing representative tumor areas, and currently no molecular markers have proven to support risk assessment of the possible presence of regional LNM in early CRC.

1.1. miRNAs in CRC progression and metastasizing

MicroRNAs (miRNAs) are a class of small (19–25 ribonucleotides) non-coding RNAs that are known to regulate gene expression at an epigenetic posttranscriptional level by acting on messenger RNA targets resulting in either degradation of the messenger RNA or translational inhibition. Individual miRNAs target multiple genes and numerous studies have shown that miRNAs are involved in cancer development, progression and metastasis through broad modulation of oncogenes and tumor suppressor genes (Iorio and Croce, 2009; de Krijger et al., 2011). Furthermore, studies of RNA on formalin-fixed paraffin-embedded (FFPE) tissues have shown that miRNAs, possibly due to their short length, are quite stable and easily accessible for tissue expression analyses in FFPE tissues (Dijkstra et al., 2012; Doleshal et al., 2008; Hasemeier et al., 2008; Siebolts et al., 2009). Profiling studies have identified specific miRNA signatures for CRC and the deregulation of several specific miRNAs have been linked to invasion and progression as well as metastatic disease and prognosis in CRC (Schetter et al., 2008; Pizzini et al., 2013; Bandres et al., 2006; Goossens-Beumer et al., 2015; Bartley et al., 2011).

In the current study we investigate tissue expression of three well known miRNAs: miR-21, miR-92a and miR-200c. Selection of the candidate miRNAs was based on review of the literature where a potential role of these three miRNAs as potential prognostic biomarkers for metastatic disease and survival in colorectal cancer is proposed (references below). Deregulated tumor expression of these miRNAs have been widely associated to invasive and migratory properties of the epithelial tumor cells, local and distant metastatic spread, advanced tumor stage and patient survival. However, there is little data on their expression in early CRCs and considering the increasing proportion of early detected cancers due to screening initiatives, it is important to explore this gap in knowledge. We analyze tumor expression levels in early CRC tissue and investigate tumor heterogeneity and the prognostic potential of these miRNAs in early stages of CRC.

miR-200c is part of the miR-200 family located on chromosome 12 (miR200c/miR-141) and chromosome 1(miR-200a/miR-200b/miR-429) and plays a key role in regulation of the epithelial-mesenchymal transition (EMT), a crucial process for metastasizing, characterized by loss of cell-cell adhesion and acquisition of mesenchymal features such as motility and the ability to invade and intravasate (Davalos et al., 2012; Park et al., 2008; Gregory et al., 2008).

Downregulation of miR-200c has been widely reported to be associated with EMT and metastatic behavior in CRC. miR-200c upregulates expression of E-cadherin and downregulates the expression of Vimentin mainly through inhibition of its main gene targets ZEB1/ZEB2, thereby enhancing the epithelial phenotype. miR-200c expression levels are reduced in the invasive margin of CRCs, and likewise CRC metastases have shown higher expression levels as an indicator of a switch on/switch off role in the reverse process mesenchymal-epithelial transition (MET) where extravasated tumor cells regain epithelial properties and proliferate in a new microenvironment (Hur et al., 2013; Paterson et al., 2013). Hur et al. found a higher miR-200c expression in non-metastatic CRC compared to metastatic cancer by qRT-PCR in a cohort mainly consisting of advanced stage CRC (Hur et al., 2013). Further, Diaz et al. found an association between high levels of miR-200c and longer overall survival as well as a possible predictive role for the miR-200 family in identifying CRC patients that are most likely to benefit from adjuvant chemotherapy (Diaz et al., 2014). Given its key regulatory role in EMT it is proposed that miR-200c could serve as a potential diagnostic and prognostic biomarker and possible therapeutic target for patients with CRC (Hur et al., 2013; Paterson et al., 2013; Diaz et al., 2014).

miR-92a belongs to the oncomir miR-17-92 cluster located on chromosome 13q31.3, a region frequently amplified in various hematopoietic and solid cancers, including CRC, predominantly leading to an upregulated expression of these miRNAs (Mogilyansky and Rigoutsos, 2013).

miR-92a functions as an oncogene in CRC by targeting the tumor suppressor PTEN (Zhang et al., 2014; Ke et al., 2014), and high levels of miR-92a has been found significantly correlated to advanced TNM stage, lymph node metastases and distant metastases in CRC (Zhou et al., 2013; Ke et al., 2014; Zhang et al., 2014). Furthermore, an association between miR-92a expression levels and overall survival has been shown (Zhou et al., 2013; Ke et al., 2014). In vitro cell studies have shown increased invasiveness, migration potential and EMT like changes in tumor cells expressing high levels of miR-92a reflected in a downregulation of E-cadherin and upregulation of vimentin and nuclear β-catenin (Zhang et al., 2014; Chen et al., 2011). Nishida et al. found significantly higher expression levels of miR-92a in micro dissected carcinoma cells and cancer associated stroma compared to corresponding normal tissues, as well as a correlation between high levels of miR-92a and lymphatic invasion, venous invasion and liver metastases, and suggest an association between miR-92a (or 17–92 cluster) and cancer progression through regulation of key components of the TGFβ pathway (SMAD2, SMAD4 and TGFBR2) (Nishida et al., 2012).

miR-21 is an oncomir located at chromosome 17q23.2 and upregulated in virtually all malignant neoplasms including CRC (Slaby et al., 2009; Volinia et al., 2006), and studies have shown a relation between altered expression levels of miR-21 and both clinicopathological features and survival in CRC (Shibuya et al., 2010). High miR-21 expression has been found related to advancing clinical UICC stage, the presence of LNM and development of distant metastases (Slaby et al., 2007; Schetter et al., 2008; Vickers et al., 2012). Additionally, associations between high levels of miR-21 and shorter recurrence free cancer specific survival in stage II colon cancer (Kjaer-Frifeldt et al., 2012, Hansen et al., 2014), poor overall survival/disease free survival (Shibuya et al., 2010; Schetter et al., 2008; Oue et al., 2014), and a poor response to adjuvant chemotherapy (Schetter et al., 2008; Oue et al., 2014) have been reported. miR-21 has been suggested as an inducer of the invasion, intravasation and metastasis process through acting on the tumor suppressor PDCD4 and also as a downstream effector in the TGF-β induced EMT process in colon cancer cell studies (Cottonham et al., 2010). Given the well documented role of miR-21 in CRC progression and metastasis predominantly in more locally advanced CRCs it is likely that miR-21 may also be an indicator of high metastatic potential in early CRC.

The current work is based on parallel studies on two independent cohorts of early CRC. With the aim of clarifying whether miR-200c, miR-21 and miR-92a could constitute prognostic markers in early CRC progression and metastasis, we measure intra-tumor expression levels of the three miRNAs in early CRC tissue in order to determine intra-tumor heterogeneity, potential intra-tumor expression gradients and finally to clarify whether expression levels could differentiate between early metastatic and non-metastatic CRC.

2. Material and methods

2.1. Heterogeneity-cohort: evaluation of intra-tumor heterogeneity

Intra-tumor heterogeneity in expression levels of the candidate miRNAs, miR-21, miR-200c, and miR-92a, was analyzed on a