Proliferation associated miRNAs -494, -205, -21 and -126

detected by *in situ* hybridization

- expression and prognostic potential in breast carcinoma patients.

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Abstract

Purpose

To visualize by *in situ* hybridization (ISH) the levels of a set of proliferation-associated miRNAs and to evaluate their impact and clinical applicability in prognostication of invasive breast carcinoma.

Methods

Tissue specimen from breast carcinoma patients were investigated for miRNAs -494, -205, -21 and -126. Prognostic associations for levels of miRNAs were analyzed based on complete clinical data and up-to 22.5-year follow-up of the patient material (n = 285). For detection of the miRNAs, an automated sensitive protocol applying *in situ* hybridization was developed.

Results

MiRNA-494 indicated prognostic value for patients with invasive breast carcinoma. Among node-negative disease reduced level of miRNA-494 predicted 8.5-fold risk of breast cancer death ($p = 0.04$). Altered levels and expression patterns of the studied miRNAs were observed in breast carcinomas as compared to benign breast tissue.

Conclusions

The present paper reports for the first time on the prognostic value of miRNA-494 in invasive breast cancer. Particularly, detection of miRNA-494 could benefit patients with node-negative breast cancer in identifying subgroups with aggressive disease. Based on our experience, the developed automatic ISH method to visualize altered levels of miRNAs -494, -205, -21 and -126 could be applied to routine pathology diagnostics providing that conditions of tissue treatment, especially fixation delays, are managed.

Keywords: breast cancer, miRNA, prognosis, proliferation, node-negative, cell cycle

Introduction

Individual treatment decisions in breast cancer rely on means to identify and manage biological and clinical tumor characteristic, patient comorbidities and the preferences of each patient. In addition to identifying aggressive disease, it is essential to develop prognostic biomarkers to recognize the increasing subgroup of breast cancer patients with favorable prognosis since these patients might be spared from chemotherapy (Duffy et al. 2015). Sustained proliferative signaling is one of the key features of malignant progression (Hanahan and Weinberg 2011). Cancer cell proliferation as assessed by Ki-67 immunohistochemistry (IHC) and mitotic counts are routine prognostic markers which have efficiently been used in subclassification of breast carcinomas, also within intrinsic subtypes (Yerushalmi et al. 2010, Healey et al. 2017). The major challenges for future include the identification of new prognostic markers for recognizing patient subgroups with different disease outcome and response to adjuvant therapies. These challenges can best be addressed with a combination of traditional clinicopathological prognostic factors and new biomarkers, such as microRNAs (miRNAs).

MiRNAs are small conserved non-coding RNA molecules participating in post-transcriptional regulation of gene expression. Aberrant miRNA expressions have been reported in several human malignancies (Chatterjee et al. 2017), including breast carcinomas (Jena 2017). MiRNAs are known for their multiple and overlapping roles in different biological processes, such as differentiation, apoptosis and cell proliferation (Ranganathan and Sivasankar 2014, Baranwal and Alahari 2010). Particularly, cell cycle regulation is known to be influenced by aberrant expressions of several miRNAs. In previous literature, abundant evidence implicates that miRNAs are major regulators of the cell cycle and proliferation, particularly at the G1 to S transition (Chivukula et al. 2008, Carleton et al. 2007).

Previously, we have shown the significant prognostic value of the protein expressions of securin (Pituitary tumor-transforming gene 1 protein, *PTTG1*) and cdc20 (Cell division cycle protein 20), regulators of the metaphase-to-anaphase transition of the cell cycle in breast cancer (Talvinen et al. 2009, Karra et al. 2014). In the present paper, we study miRNAs -494, -205, -21 and -126 which, based on literature, are involved in cell cycle regulation with suggested roles in proliferation. In

light of previous literature, miRNA-494 (He et al. 2014) has been suggested with a global regulatory role in cell cycle progression, including participating in regulation of *PTTG1* and CDC20 (Yamanaka et al. 2012) although there are no previous reports to be found on detecting miRNA-494 by *in situ* hybridization (ISH) in breast cancer. MiRNA-205 has been reported to inhibit proliferation in triple-negative breast carcinoma (TNBC) cell lines (Piovan et al. 2012). MiRNA-21 has been described to promote the cell cycle by targeting tumor suppressor genes and resulting in increased proliferation in both the malignant epithelium and the adjacent stroma of breast carcinomas (Zhang et al. 2016). Previous literature provides convincing evidence on the involvement of miRNA-126 in inhibiting G1/G0 to S-phase (Zhang et al. 2008). Up-regulation of miRNA-126 has been verified to inhibit proliferation in several carcinomas, including breast cancer (Ebrahimi et al. 2014, Tavazoie et al. 2008). Also, the therapeutic potential of miRNAs, including -21, -126, -205 and -494 has been discussed (Kaboli et al. 2015, Lim et al. 2014).

In the present study, we have optimized and automated a sensitive protocol for detecting a set of proliferation-associated miRNAs. The method has been verified in detecting miRNAs -21 and -126 and thereafter applied for studying miRNAs -494 and -205 which have previously not been established with prognostic associations in breast cancer. Expression patterns of the miRNAs are detected for a maximum of 304 cases and prognostic associations presented among 285 patients with invasive breast carcinomas. All evaluated tissue specimen have been included in the study only after verifying the quality of RNA. Interestingly, microRNA-494-ISH – which has not previously been reported for breast carcinoma – showed prognostic impact, particularly among node-negative breast carcinomas, as analyzed based on up to 22.5 years of follow-up.

Materials and methods

Patients and specimen

The study is based on four separate sets of breast carcinoma specimen (Materials I – IV). The first set (Material I) comprises fresh specimen of both benign breast tissue and breast carcinomas treated with different fixation conditions (n = 9, 4 normal and 5 breast carcinoma specimen). The second set (Material II) was used to demonstrate different expression patterns of the studied miRNAs in normal breast tissue and in invasive breast carcinoma (n = 25, 6 normal, 6 luminal, 7 *Her2*-amplified and 6 triple-negative breast carcinoma specimen). The remaining two sets (Materials III

and IV) comprised breast carcinomas used for detecting expression patterns of selected miRNAs in a clinical material to determine their clinico-pathological and/or prognostic associations (Table 1).

In more detail, Material I was collected fresh from the operation theatre of Turku University Hospital, Turku, Finland, in 2016. All specimen were used to systematically evaluate the integrity of miRNA on basis of small nuclear RNA U6. For this purpose, the fresh tissue specimen were divided into several representative pieces and each piece was exposed to different fixation delays according to a protocol (no delay, 1 hour, 4 hours, 1 – 7 days) and fixation times (24 or >48 hours). After fixation, the intensity levels of U6 were evaluated under light microscope for each piece of benign and malignant breast tissue of the fixation experiment. In all specimen, the intensity of U6 was scored 0 – 3+ (NG, PK) (Fig. 1A). This score was further used to allocate the specimen into groups representing adequate (2 – 3+) as opposed to inadequate (0 – 1+) RNA integrity. According to U6 detection, acceptable quality of RNA was observed when formalin fixation was started within 2 days after surgery of breast carcinoma (Fig. 1B) and even more than 2 days in benign breast tissue. In our experience, duration of formalin fixation (24 – 72h) did not influence the quality of RNA, as concluded from the U6 detections. The above described principle was further applied to the archival patient materials (Materials II – IV) so that each patient case was included in the analyses only after the integrity of RNA was ensured based on acceptable U6 level (NG).

Material II was used to demonstrate the different expression patterns of the studied miRNAs in patients diagnosed with invasive breast carcinomas in Turku University Hospital, Turku, Finland, during 2016. In addition, the material included specimen of benign breast tissue from breast reductions. These specimen were used to register the expression patterns of the studied miRNAs in benign and malignant epithelium and reactive stroma and endothelial cells surrounding the tumor.

Selected miRNAs were further analyzed for clinico-pathological and prognostic correlations in invasive breast carcinomas separately for all intrinsic subtypes, and for TNBCs (Materials III and IV, respectively). After verifying the quality of miRNA based on U6-positivity, as described above, a maximum of 285 invasive breast carcinomas were available for clinical and prognostic correlations. These patients were diagnosed with unilateral, primary breast carcinomas in Central Finland Central Hospital, Jyväskylä, Finland during 1987 – 1997 (Material III) and in Turku University Hospital, Turku, Finland during 2005 – 2015 (Material IV). All patients were treated with surgical resection or mastectomy with a sentinel node biopsy and, in case of metastatic disease, axillary evacuation. Postoperative radiation therapy and adjuvant treatments were conducted according to the international guidelines for breast cancer classification and treatment at the time of

the diagnosis (Goldhirsch et al. 2009). No preoperative adjuvant treatments were conducted. Complete clinical follow-up information was available either from pathology reports and patient files or from Auria Biobank, Turku University Hospital, Turku, Finland. The follow-up data included the established prognosticators of clinical breast cancer treatment, i.e. axillary lymph node status, tumor size, histological grade, Ki-67 and the intrinsic classification defined according to the surrogate guidelines by the 12th St Gallen International Breast Cancer Conference (2011) Expert Panel (Goldhirsch et al. 2011). Causes of death were obtained from autopsy reports, death certificates and from Finnish Cancer Registry. The maximum follow-up periods for prognostic associations were 22 years and 6 months (mean 9.6 years).

The selection of the patient material and the study setting was approved by The Ethics Committees of Central Finland Central Hospital and Turku University Hospital, and Auria Biobank.

Methods

Apart from the experimental fixation protocols applied for Material I, all other specimen (Materials II – IV) were prepared according to the standard practice of a clinical histopathology laboratory, i.e. fixed in buffered formalin (pH 7.0) and embedded in paraffin. The specimen were collected in tissue microarrays (TMAs) prepared by punching the paraffin block of each tumor with either a 4 mm (Material II), 0.6 mm (Material III, Karra et al. 2012) or 1.5 mm (Material IV, Repo et al. 2017) diameter cylinder.

***In situ* hybridization**

All treatments of tissue samples were done RNase-free. Tissues were sectioned onto positively charged slides at 3 – 4 μm thickness and stored at 8 °C until used (within three weeks). On the previous day, the slides were baked at 60 °C for 3 h and stored then overnight at 8 °C. Deparaffinization and hydration were done manually in descending scales of xylene and ethanol. The slides were loaded wet into Ventana Discovery XT automated slide staining instrument (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA). The bulk solutions included Discovery EZ Prep Solution, Liquid Coverslip, RiboWash and Reaction Buffer (Roche/Ventana). Double-digoxigenin (DIG) labeled LNA-modified probes were designed by Exiqon (Exiqon A/S, Vedbaek, Denmark) and treated according to the recommendations by the manufacturer. Following probes were applied: U6 snRNA positive control (99002-15), scrambled microRNA negative control (99004-15), hsa-miR-21-5p (38102-15), hsa-miR-126-3p (from microRNA ISH Optimization Kit 5, 90005), hsa-miR-494-3p (616987-360) and hsa-miR-205-5p (18099-15). All steps of hybridization

and fluorescent detection were optimized for each miRNA separately in order to achieve best possible signal to background ratio. Research ISH Blue/Red procedure was used in the hybridization. Tissue permeabilization was optimized for breast tissue under study and finally performed treating the samples for 20 min at 37 °C applying ISH Protease 3 (Roche/Ventana). Probes were diluted in 2x Formamide-free microRNA ISH buffer (Exiqon, 90000) to the following concentrations U6 0.3 nM, scrambled probe and miRNA-126 3.0 nM, miRNAs -21 and -494 1.5 nM, and miRNA-205 1.8 nM and, finally, applied onto the slides in volume of 200 µl. The slides were hybridized for two hours and washed using software option for stringency wash 0.1x SSC at the following optimized probe specific temperatures: 53 °C (U6, scrambled probe and miRNA-21), 56 °C (miRNA-126), 54 °C (miRNA-494) and 55 °C (miRNA-205).

Immunofluorescent detection

For the fluorescent detection of the probes, RUO DISCOVERY IF procedure was used applying Discovery Inhibitor (Roche/Ventana), Blocking Reagent (Roche Diagnostics, Mannheim, Germany), anti-DIG antibody (Roche Diagnostics, clone 1.71.256) and OmniMap anti-ms HRP (Roche/Ventana). Anti-DIG was diluted 1:200 and 200 µl was applied onto each slide. Amp HQ Kit and Amplification anti-HQ HRP Multimer (Roche/Ventana) were used to amplify the signal before applying rhodamine substrate (Roche/Ventana). Amplification was not needed for U6. After immunofluorescent detection slides were collected from the instrument and mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes by Life Technologies, Eugene, OR, USA) following the manufacturer's instructions. All specimen were studied with a microscope (Axio Scope A1, Carl Zeiss Microscopy GmbH, Jena, Germany) for determining intensities for U6 and the studied miRNAs. Thereafter, the slides were scanned using automatic exposure time for TRITC and DAPI channels (Pannoramic Midi FL slide scanner, 3DHISTECH Ltd., Budapest, Hungary) and Pannoramic Midi and Pannoramic Viewer (3DHISTECH Ltd.) and in order to produce representative images of expression patterns of the miRNAs.

Interpretation of ISH

After verifying the quality of each specimen, we detected miRNAs -21, -126, -494 and -205 by ISH (Materials II – IV). MiRNAs -21 and -126 could be used to verify the appropriate pattern of positivity similar to that previously published for breast cancer. Each miRNA showed a distinct pattern of expression which was registered separately for cancer cells and stromal cells, and in case

of miRNA-126, for endothelial cells. The scrambled probe showed no signal. All miRNAs were classified either negative (intensity 0) or positive (intensities 1 – 3+).

Immunohistochemistry

IHC was performed on sections cut at 3.5 µm. For securin and cdc20, IHC and interpretations were performed as previously presented (Karra et al. 2012, Karra et al. 2014). For cyclinB1 (Abcam, ab32053, clone Y106), we used an automated immunostaining machine Discovery XT (Roche Diagnostics/Ventana Medical Systems). Deparaffinization, epitope retrieval (standard option, Cell Conditioning 1, Roche/Ventana) and primary antibody incubation (dilution 1:100, 40 min at 37 °C) were done on the platform. OmniMap HRP and ChromoMap DAB Kit (Roche/Ventana) were applied for detection. CyclinB1-IHC was evaluated as fraction (%) of positively stained invasive cancer cell nuclei and/or cytoplasm (0 vs <10% vs ≥10%). IHC and interpretations for estrogen and progesterone receptors and Her2, and *HER2*-SISH were performed according to guidelines and principles of clinical pathology routine as previously described (Gurvits et al. 2016).

Statistical analysis

Associations between categorical variables and the studied miRNAs were analyzed using contingency tables and Fisher's exact test. Survival analyses were done in Material III for the whole data and also for subsets of the data split by nodal status, intrinsic classification, histological grading (grades I – II vs III), tumor size (≤2cm vs >2cm) and Ki-67 (≤ 14% vs >14%) and in Material IV for nodal status. Survival analyses were first started with univariate analysis for time to breast cancer-specific death, estimated using the Kaplan-Meier technique and log-rank tests. Thereafter, the differences between categorized values were tested using the Wilcoxon test. Hazard ratios with 95% confidence intervals were calculated to quantify differences between positivity vs negativity of the studied miRNA. For further analysis involving more complicated models with multiple factors, we evaluated the studied miRNAs and the established prognosticators of breast cancer i.e. nodal status, tumour size, histological grading, intrinsic classification and proliferation as expressed in Ki-67- immunopositivity. Restricted means of 5 and 20 years survival time were also used to describe differences. P-values under 0.05 were considered significant. Patients with missing data were censored. Statistical analyses were performed using SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA) and R Statistical Software (R Development Core Team, 2017, and Terry M Therneau, Thomas Lumley).

Results

To begin with, the different expression patterns of miRNAs -21 and -126 were registered (Material II) (Fig. 2). MiRNA-21 was negative in benign breast epithelial cells and occasionally positive in single stromal cells. Instead, miRNA-21 was variably expressed in malignant epithelial cells and cancer-associated fibroblast-like cells of breast carcinoma. We observed no difference in miRNA-21 expression among specimen representing the different intrinsic types of breast carcinomas. MiRNA-126 was observed in endothelial cells both in benign and malignant breast tissue. MiRNA-205 was positive in basal epithelial/myoepithelial cells of normal mammary ducts but negative in luminal epithelial cells of normal breast tissue and in most cases of breast carcinomas. The few carcinoma specimen showing miRNA-205-positivity represented subtypes of *Her*2-amplified and TN breast cancer. MiRNA-494 was expressed with varying intensity in all normal tissues. In breast carcinomas, both miRNA-494-positive and negative specimen were observed while the negative specimen represented luminal and *Her2*-amplified subtypes.

Clinical materials (III and IV) were evaluated for miRNAs -494 and -205. Material III was classified into subgroups showing negative (intensity 0) vs positive (intensities 1 – 3+) miRNA expression. Positive miRNA-494 was detected in 30.2 % of breast carcinomas (n = 245). No associations were detected between miRNA-494-expression and clinical features including axillary lymph node status, tumor size, grade or proliferative activity of the tumors. In prognostic analyses (n = 233), however, we observed a trend for unfavorable course of disease for patients with miRNA-494-negativity as opposed to miRNA-positivity (Fig. 3). Among node-negative patients (n = 126), miRNA-494-negativity was associated with breast cancer outcome predicting 8.5-fold risk of breast death (p = 0.04, CI 1.1 – 62.5). The same trend was seen for low grade (I – II) carcinomas where miRNA 494-negativity was detected for patients with an unfavorable outcome although the association failed to show statistical significance (p = 0.07). In analyses involving proliferation marker Ki-67 (<14% vs ≥ 14 % of cancer cells) and the cell-cycle regulators cyclinB1, securin and cdc20, miRNA-494 showed statistically significant association with cdc20 (Chi square 0.009). In multivariate analysis involving nodal status, intrinsic classification and Ki-67 among all breast carcinomas (n=166), miRNA-494-negativity showed independent prognostic value (p = 0.04, HR 2.1, CI 1.0 – 4.1). Instead, miRNA-494 sparsely failed to show significant prognostic value among breast carcinomas classified according to tumor size (≤20mm vs >20mm), histological grade (grades I – II vs III) or both (p-values for miRNA-494 0.05, 0.05 and 0.07, respectively). Among node-negative patients, miRNA-494-negativity (n = 97) was and independent prognosticator for

breast cancer death even among patient subgroups representing different intrinsic classification (p = 0.03, HR 10.1 CI 1.3 – 76.9) or tumor size (p = 0.03, HR 9.7, CI 1.2 – 76.9). MiRNA-205 was observed in the tumor cells in only in 9 cases of the studied 203 breast carcinomas. Single positive cases represented luminal and *Her2*-amplified carcinomas (fraction of miRNA-205 positive cases 6% and 3% among luminal and *Her2*-amplified carcinomas, respectively). Clinical characteristics of the miRNA-205-positive cases indicated favorable course of disease with a minimum breast cancer-specific survival time of 12 years.

In TNBCs (Material IV), the expression of miRNA-494 was heterogeneous and, therefore, we performed a more detailed assessment where cancer cell areas with varying miRNA-494 expressions were evaluated separately. This evaluation principle allowed us to detect even also those cases where miRNA-494 expression was only partly or focally lost. Consequently, 67.5% of TNBC patients showed at least partial loss of miRNA-494. Loss of miRNA-494 showed a trend for prognostic associations among node-negative patients although the correlation was not statistically significant, probably due to the small patient material (p = 0.2, n = 25) (Fig. 3). Among the clinico-pathological features, miRNA-494-negativity showed association with tumor size (Fisher's exact test 0.03) but not with nodal status. Finally, when evaluating the associations of miRNA-494 with cell cycle related proteins cyclinB1, securin and cdc20, statistically significant association was observed with securin (Fisher's exact test 0.02). No miRNA-205 expression was observed in TNBC. The summarized results of the miRNAs -21, -126, -205 and -494 are presented in Table 2.

Discussion

In our results based on up-to 22.5-year follow-up, reduced level of miRNA-494 indicated unfavorable course of disease. Previous literature on the prognostic role of miRNA-494 is sparse and partly controversial. Similar to our results, reduced levels of miRNA-494 have been associated with poor prognostic features in epithelial ovarian carcinomas, pancreatic and gastric carcinomas (Yang et al. 2017, Ma et al. 2015, He et al. 2014). On the contrary, increased levels of miRNA-494 have been associated with unfavorable outcome in colorectal, breast and ovarian cancers (Sun et al. 2014, Marino et al. 2014, Yang et al. 2015). The variable prognostic associations observed in different malignancies may reflect the various biological roles of miRNA-494. Particularly, aberrant miRNA-494 expression has been has been reported to influence cell cycle regulation. In cholangiocarcinomas, it has been shown that miRNA-494 induces an arrest at G_2/M by modulating the protein levels of several genes, including cyclinB1, securin and cdc20, and by directly down-

regulating PTTG1 (Yamanaka et al. 2012). Also our results indicated an association between miRNA-494 and cdc20, a regulator of metaphase-anaphase transition involved in triggering sister chromatid separation (Kapanidou et al. 2017). In different malignancies, however, miRNA-494 has been described to both promote (Liu et al. 2015) and inhibit proliferation (Zhao et al. 2016, Kim et al. 2011). In our prognostic analyses, reduced level of miRNA-494 predicted considerably increased risk of breast cancer death in node-negative disease (HR 8.5, p = 0.04, CI 1.1 – 62.5). Even based on long-term follow-up (20 years from diagnosis) we observed a considerable survival difference between patients with negative vs positive miRNA-494 (16.8 vs 19.7 years, respectively). In literature, node-negativity is among the strongest predictors for breast cancer survival (Nicolini et al. 2017). In our experience, miRNA-494-detection could be applied in node-negative breast cancer to identify a patient subset with potentially aggressive course of disease and provide additional evidence to favor adjuvant therapy.

Literature on the role of miRNA-205 in human tumors is complex and controversial suggesting various aberrations in different human malignancies as compared to their normal counterparts (Zhang et al. 2015). The majority of studies suggest that miRNA-205 is a tumor suppressor (Hulf et al. 2013, Hou et al. 2013, Hagman 2013, Hanna et al. 2012, Childs et al. 2009) while some studies present miRNA-205 as an oncogene (Kalogirou et al. 2013, Karaayvaz et al. 2013). There are few papers where ISH-detected miRNA-205 has been associated with survival of breast carcinoma patients (Quesne et al. 2012, Sempere et al. 2007). Also, the data on miRNA-205 over- vs up-regulation in breast cancer subtypes is controversial (Greene et al. 2010). In our material, the great majority of cases lacked miRNA-205 although we observed increased level of miRNA-205 in single cases of luminal and *Her*2-amplified breast carcinomas. The small number of cases with aberrant level of miRNA-205 encountered in our material did not allow prognostic conclusions. In literature, however, miRNA-205 has been associated with progression of breast cancer (Markou et al. 2014). Loss of miRNA-205 has also been associated with reduced overall and metastasis-free survival in breast carcinoma (Quesne et al. 2012, Huo et al. 2016) although most papers evaluating the prognostic associations of miRNA-205 report variable results from small and heterogeneous materials.

The observed localizations and expression patterns of miRNAs -21 and -126 in our material were similar to previous literature. Both in the literature and our findings, levels of miRNA-21 are increased from precancerous to malignant breast disease and expressed variably in epithelial cells

and in tissue-associated fibroblasts (Sempere et al. 2007, Rask et al. 2011, Qi et al. 2009, Hug et al. 2015, Nielsen et al. 2014). According to literature, miRNA-126 has been observed in endothelial cells where expression changes have been suggested to reflect differences in the distribution of blood vessels and in the integrity and/or structure of the neovasculature (Jørgensen et al. 2010, Sempere et al. 2010). Previous literature based on PCR detections has shown reduced expression of miRNA-205 as compared to non-malignant breast tissue (Song et al. 2015, Haga et al. 2012). Similar to our results (Table 2), previous literature emphasizes the heterogeneity of miRNA-205 in breast carcinoma reporting increased levels in all intrinsic subtypes (Greene et al. 2010). MiRNA-494 has not previously been reported in breast cancer using ISH.

In previous literature, numerous methods have been used for detecting miRNAs, and the achieved results appear to vary depending on the used technique. The practical improvement described in the present paper is the development of an automatic ISH method whereas, in previous literature, only few investigations report using fully automatized ISH for miRNA detection (MacKenzie et al. 2014, Sempere 2014, Singh et al. 2014). The special advantage of ISH is enabling visual evaluation of the miRNAs in different cell types and localizations (Chugh and Dittmer 2012, Pritchard et al. 2012, Urbanek et al. 2015). In addition to the detection method, the size and construction of tissue specimen as well as alterations in tissue processing cause variations in the results. In the present study, only cases with acceptable U6 signal were qualified for analysis. This meant that the presented results were obtained after excluding 42 % of the tissue cores in the original set of specimen on basis of unacceptable U6. In the present paper, different conditions of tissue processing were tested for delay and time of fixation. The results emphasize the influence of fixation delay on the conservation of miRNA. Our results from U6 detection show that no longer than two-day delay from removal of the surgical specimen to the initiation of formalin fixation will result in acceptable quality of miRNA in breast cancer tissue. Presumably, large size of resected tissue material also delays the fixation in the center of the specimen leading to deterioration of miRNA quality. Contrary to the report by Singh et al. (2014), in light of our observations, the length of fixation did not influence the conservation of miRNA. We conclude that the used automatized ISH method is well applicable to use in routine pathology laboratory. However, in our experience also other aspects of tissue fixation than only fixation delay and length should be considered when clinical materials are used for miRNA detection. This is especially critical when miRNA detection is applied on archived tumor material where tissue collection and fixation conditions cannot be

standardized. If miRNA detection is considered for routine use in prognostication of individual patients the conditions for tissue processing should be carefully standardized.

In summary, the applied automatic ISH method is well applicable for detection of altered miRNA levels and could be incorporated into routine pathology diagnostics. Still, special caution is required when miRNA detection is applied as part of the prognostic assessments of an individual patient since conditions of tissue treatments, especially fixation delays may significantly influence the results. As a new finding from our material, reduced level of miRNA-494 indicated unfavorable course of disease suggesting that miRNA-494 could be applied in predicting the outcome of invasive breast cancer. Particularly, miRNA-494 provided additional prognostic evidence for a subgroup of node-negative patients with unfavorable course of disease and who could benefit from adjuvant treatments.

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Legends for the Figures

Figure 1. A. Examples of U6 detections in breast carcinomas graded 0 - 3+. Specimen with U6 corresponding to grades 2+ and 3+ were considered acceptable for further evaluation of miRNAs. B. The effect of fixation conditions on specimen of fresh breast tissue were tested in series of immediate to delayed fixation (1 day – up-to 7 days delay). According to U6 detection, acceptable quality of RNA in breast cancer tissue was observed when formalin fixation was started within 2 days after surgery and continued for at least 24 hours. (Magnification x400, scale bar 100 μm).

Figure 2. Examples of expression patterns of miRNAs -494, -205, -126 and -21. The Figure shows for each miRNA the normal expression patterns in benign breast and the aberrant expression patterns in the epithelial and stromal compartments of invasive breast carcinomas. In addition, the Figure presents separately the expression patterns observed in the majority of breast carcinoma cases as opposed to exceptional expression patterns in the minority of breast carcinoma cases. (Magnification x400, scale bar 100 μm).

Figure 3. Kaplan-Meier curves showing disease-specific survival of patients with miRNA-494 negative vs positive breast carcinomas. The survival curves are shown separately for all subtypes of carcinomas (A) as well as for subgroups of node-negative (B), low-grade (histological grades I – II) (C) and triple-negative node-negative (D) disease. Statistically significant survival difference was observed among patients with node-negative breast carcinomas although decreased miRNA-494 levels showed a trend for worsened survival also in the other analyses.

Compliance with Ethical Standards

Funding: The study was supported by grants from Cancer Society of South-West Finland, Foundation of Finska Läkaresällskapet and Turku University Hospital.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The work has been approved by the ethics committees of Turku University Hospital and Central Hospital of Central Finland, Turku, Finland (permit numbers 0286/2002, 7765-2002 and AB159859). All patient material has been obtained from archives of biobanks and includes written consent from patients obtained in accordance to the ethics permits and the Finnish Biobank Act (688/2012).

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