ANALYSIS OF VEGF CIRCULATING RNA ISOFORMS IN PATIENTS WITH BREAST CANCER

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The present study aims to estimate and compare the levels of cell-free circulating RNAs of three interleukins IL-6, IL-8, and IL-18 and three splice variants of the vascular endothelial growth factor (VEGF), namely 121, 165 and 189, in blood plasma of patients with stage I / II breast cancer and healthy controls. The study reveals that patients with breast cancer have significantly elevated levels of circulating VEGF121 and VEGF165 RNAs, so far unreported in the literature. We also confirm that levels of circulating IL-8 and IL-18 RNAs are considerably increased in breast cancer patients.

Keywords: circulating RNA, VEGF isoform, breast cancer, cytokines, qPCR

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Although circulating nucleic acids (DNA and RNA) have long been proposed as diagnostic and prognostic markers of pathology [1–4], they are still far from exhausting their diagnostic potential. Found in human blood plasma, they are easily accessible for analysis. Given that the circulation continuously and vigorously “monitors” the state of bodily organs and tissues, abnormalities in blood composition can indicate pathology developing anywhere in the body. The amount of DNA per cell is quite stable in different tissues, but the transcript profile is unique for every cell type, which may be useful in developing new diagnostic methods.

The presence of cell-free circulating RNA (cfRNA) in the circulation can be linked to different events, such as necrosis, apoptosis, or active metabolic secretion [5–8]. PCR-based quantification of RNA found in the plasma is a routine and relatively cheap technique that can become a convenient diagnostic screening tool in case new clinically relevant biomarkers are discovered.

Breast cancer is the most common cancer affecting women. It accounts for 16 % of all new cancer cases in females [9]. There is abundant evidence in the literature indicating changes in cfRNA levels in cancer patients [1, 2]. Tumor
form formation is mediated by cytokines and factors of cell growth and differentiation. Cytokines play an important role in both inducing breast cancer and inhibiting its progression [10, 11].

Vascular endothelial growth factor (VEGF) is one of the key proteins stimulating formation of blood vessels and thus contributing to tumor growth. Tumors rely on angiogenesis to keep up oxygen supply as they grow and to spread hematogenously. Cytokines and growth factors secreted by tumor and stromal cells stimulate proliferation of endothelial cells. VEGF is strongly associated with increased tumor aggressiveness and metastasis [12–14]. VEGF levels are increased in the serum of cancer patients. There is evidence that circulating levels of VEGF may be a surrogate marker of angiogenesis and/or metastasis [15]. Abnormal angiogenesis is typical for many types of cancer, but roles of different VEGF isoforms involved in this process vary. Using an experimental breast tumor model, it was shown that VEGF-121 is the most carcinogenic isoform [16]. Expression of VEGF-121 is increased in comparison with VEGF-165 in patients with colorectal and prostate cancers [17, 18].

Previously, we showed that levels of IL-8 and IL-18 cfRNAs increase in the early stages of breast cancer, while levels of IL-6 cfRNA remain unchanged [19]. The aim of this study was to estimate cfRNA concentrations of three most abundant VEGF isoforms, namely VEGF-121, VEGF-165 and VEGF-189, in patients with early stages of breast cancer and to corroborate previously obtained results for IL-6, IL-8 and IL-18.

METHODS

The study was carried out in 36 women between 34 and 81 years of age (mean age of 67.9 years) with histologically confirmed breast cancer. Of all the participants, 2 had stage 0 cancer (TisN0M0), 17 had stage I cancer (T1N0M0), 13 had stage II (7 patients with T1N1M0 and 6 patients with T2N0M0), 2 patients had stage III (T2N1M0), 1 patient had stage IIA (T2N2M0), and 1 patient had stage IIB (T2N3M0). Thirty-two participants had tumors as large as >2 cm. Regional lymph node metastases were found in 11 patients, with cancer spreading to 1–3 lymph nodes in 9 females and to 4 and more lymph nodes — in 2 females. Non-specific ductal carcinoma was the most common cancer type in our study (21 women); lobular cancer was found in 4 patients, specific cancer types — in 9 patients, intraductal lesions — in 2 patients. Tumor grade distribution was as follows: 6 cases of grade I, 16 cases of grade II, and 14 cases of grade III. None of the patients included in the study received preoperative anticancer therapy.

The control group included 56 healthy women aged 24 to 55 years, mean age being 40 years.

Participants’ data were anonymized. The study was approved by the local ethics committee (Protocol No. 2016/67).

Blood samples were collected before the surgery into disposable EDTA-containing BD Vacutainer tubes (Becton, Dickinson and Company, USA) and transported to the laboratory at room temperature within 30 min. To obtain plasma, 1 ml of blood was placed into a 1.5-ml polypropylene tube and centrifuged at 1,000 rpm for 10 min. The supernatant was then transferred to a new clean tube and centrifuged at 3,000 rpm for 10 min. Then the upper fraction (plasma) was transferred to new tubes and stored at −70 °C for no longer than 10 days. Extraction of cfRNA was performed using the PROBA-NK kit by DNA-Technology, Russia, according to the manufacturer’s protocol. Purified cfRNA was immediately used in the reverse transcription reaction with specific RT-primer as suggested by the standard protocol. Complementary DNA was either immediately used for PCR or stored at −20 °C for <10 days.

To measure cfRNA levels, quantitative RT-PCR was performed using ImmunoGenetics (a commercial kit by DNA-Technology, Russia) according to the manufacturer’s protocol and the DTprime amplifier by the same manufacturer.

The GUSB transcript β-glucuronidase was used as a reference, since GUSB does not change its expression in cancer [20, 21]. PCR data were normalized using the ∆∆Ct method [22]. Significance of differences was estimated by Student’s t-test. The difference was considered significant at p < 0.05.

RESULTS

The figure below shows expression levels of the studied genes coding for IL-6, IL-8, IL-18, VEGF-121, VEGF-165, and VEGF-189 and the reference gene GUSB in plasma of patients with breast cancer and the controls.

RNA levels in plasma of patients with breast cancer and the controls. The X-axis shows names of the studied transcripts; the Y-axis shows the amount of cfRNA, OU

* — p < 0.05
Significant differences in cfRNA levels were observed between the experimental and the control groups for IL-8, IL-18, VEGF-121 and VEGF-165 (p < 0.05). Although the levels of IL-6 and VEGF-189 cfRNAs were slightly increased in the experimental group compared to the controls, the difference was not significant.

DISCUSSION

The obtained results are consistent with [19] where relative amounts of IL-8 and IL-18 RNAs were significantly increased in patients with different stages of breast cancer in comparison with healthy controls.

Significantly increased levels of circulating RNA of VEGF-121 and VEGF-165 isoforms in patients with breast cancer are also consistent with the results of other studies focused on the expression of these VEGF variants in cancer [12–18]. Tokunaga et al. classified patterns of VEGF mRNA found in human tumors into three types: type 1 expression, VEGF-121 only; type 2 expression, VEGF-121 and VEGF-165; type 3 expression, VEGF-121, VEGF-165 and VEGF-189 [23]. Type 3 expression (a combination of three isoforms) is found in rectal cancer metastases [23], renal cell carcinoma [24], hepatocellular carcinoma [25] and non-small-cell lung cancer with poor prognosis [26]. Some authors believe that VEGF-189 activates an autocrine proliferation loop in breast cancer through semaphoring receptors (specifically, through Neuropilin-1) [27]. On the whole, our findings support Tokunaga’s hypothesis, showing the presence of either VEGF-121, or a combination of VEGF-121 with VEGF-165 and/or VEGF-189 in the circulation.

CONCLUSIONS

This study corroborates the results of our previous work revealing increased blood plasma levels of IL-8 and IL-18 transcripts in patients with stage I and II breast cancer. The patients were found to have increased levels of VEGF-121 and VEGF-165 cfRNAs. Our findings support Tokunaga’s hypothesis about the associations between the circulating RNA levels of basic VEGF isoforms (VEGF-121 and VEGF-165) and tumor growth and between VEGF-189 and metastasis.

References


