

Soluble VCAM-1 and E-selectin in breast cancer: relationship with staging and with the detection of circulating cancer cells*

H.C. SILVA¹, F. GARCAO², E.C. COUTINHO¹, C.F. DE OLIVEIRA³, F.J. REGATEIRO¹

¹Department of Genetics, e-mail: hcoimbra@ci.uc.pt, Coimbra University Medical School, 3004-504 Coimbra, Portugal; ²Department of Immunology Coimbra University Medical School, and ³Department of Gynecology, Coimbra University Hospital, Coimbra, Portugal

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In breast cancer, the correct evaluation of cancer dissemination is essential to establish prognosis and treatment choices. This study analyses the relationship between circulating levels of soluble VCAM-1 and E-selectin and the presence of circulating cancer cells in breast cancer patients.

Plasma levels of VCAM-1 and E-selectin were measured by enzyme-linked immunosorbent assay (ELISA). The presence of circulating cancer cells was diagnosed using a RT nested-PCR assay detecting the cancer specific transcript, epidermal growth factor receptor variant III (EGFRvIII) mRNA. Blood samples were collected from 64 patients divided in three groups: group A of 11 women selected for neoadjuvant chemotherapy; group B of 13 women with metastatic disease and group C, with 40 women having completed their treatment at least one year ago and with no evidence of relapse. The mutant transcript was detected in 45.5% of patients from group A, in 61.5% of patients from group B and in none of the group C patients. For both VCAM-1 and E-selectin, plasma levels increased with disease staging and with the presence of EGFRvIII mRNA in peripheral blood. The differences were statistically significant ($p < 0.025$) when group C was compared with all patients from group B, with patients from group B with EGFRvIII positive results or with all patients with EGFRvIII positive results.

Increased plasma levels of VCAM-1 and E-selectin are associated with advanced stage of breast cancer and with the presence of circulating cancer cells. The combined analysis of these parameters may contribute to a more accurate evaluation of cancer dissemination.

Key words: breast cancer, VCAM-1, E-selectin, EGFRvIII, occult disseminated disease

Breast cancer is the second leading cause of cancer death in most countries. In the last decades, prognosis has improved mainly due to more widespread use of systemic therapy in high risk patients. Nevertheless, metastasis will often dictate a fatal course. Identification of accurate factors predicting relapse and the diagnosis of occult dissemination are very important to optimize therapeutic choices.

Intercellular adhesion molecules are intimately involved in inflammatory reactions. They also seem to play an important role in cancer progression, namely in neoangiogenesis, invasion and metastasis [1–10]. Vascular cell adhesion molecule-1 (VCAM-1 or also, CD106) is a transmembrane glyco-

protein of the immunoglobulin-like superfamily that serves as endothelial ligand for integrins [11]. Endothelial selectin (E-selectin or CD62E), also known as endothelial leukocyte adhesion molecule-1 (ELAM-1), is one of the three members of the selectin adhesion molecules. This transmembrane protein binds cells expressing specific ligands containing sialyl-Lewis residues [12]. Both VCAM-1 and E-selectin are expressed in endothelial cells activated by cytokines via a NF- κ B dependent pathway and are implicated in lymphocyte trafficking. Elevated serum concentrations of soluble forms of these molecules have been described in patients with solid tumours, including breast cancer, colorectal cancer, melanoma, gastric cancer and prostate cancer [1]. In breast cancer, serum levels of adhesion molecules have been correlated with disease progression and are proposed as markers of angiogenesis and as prognostic factors [1–10].

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In breast cancer, the diagnosis of circulating cancer cells has been associated with recurrence and poor survival both in early and advanced disease [13–15]. The most common mutant of epidermal growth factor receptor (EGFR), the epidermal growth factor receptor variant III (EGFRvIII), is a constitutively active receptor involved in breast carcinogenesis and metastasis [16–18]. The mutant results from an in-frame deletion of the coding exons 2-7, corresponding to a deletion of 801 bp from the mRNA. It is a cancer specific marker, described in a wide variety of human malignancies, including up to 50–78% of breast cancers and not expressed by blood cells or other normal tissues [19–20]. Our group has been studying the problem if these unique characteristics could promote EGFRvIII a candidate marker for the diagnosis of breast cancer occult dissemination.

In this study, we assessed the correlation between plasma concentrations of E-selectin and VCAM-1 and the presence of blood circulating cancer cells in breast cancer patients.

Patients and methods

Patients and samples. Blood samples from 64 breast cancer patients, divided in three groups, were studied: group A, with 11 women of stage II or III (FIGO classification), mean age 48 years (± 12.6), selected for neoadjuvant chemotherapy; group B, with 13 women with metastatic disease (FIGO stage IV), mean age 56.7 years (± 17.6) and group C, with 40 women, mean age 57.6 years (± 11.8), having completed their treatment at least one year ago and with no evidence of relapse. In group A and in 4 patients from group B blood samples were collected prior to any treatment, all other patients from group B had already been submitted to chemotherapy and had finished treatment at least one month ago. Women in group C were at stages I, II or III at the time of diagnosis. According to international recommendations, follow-up included clinical surveillance, mammography of the remaining breast, routine blood tests and serum determination of CA15.3. Patients were undergoing treatment in the Gynaecological Department of Coimbra University Hospital. All patients gave their informed consent.

Ten milliliters of venous blood were collected and nucleated cells were separated by gradient density centrifugation with Ficoll. A total of 5×10^6 – 1×10^7 viable cells were used for RNA extraction and one milliliter of plasma was collected and kept at -80°C until use.

Cell lines. U87MGEGFR, a glioblastoma cell line expressing EGFRvIII and not EGFR, was generously provided by Professor Wong from the Ludwig Institute for Cancer Research (La Jolla, San Diego); it was maintained in DMEM supplemented with 10% FBS and 200 $\mu\text{g/ml}$ of Geneticin. CTLL-2, a lymphoblastic cell line, was a generous gift from the Histocompatibility Center of Coimbra; cells were cultured in RPMI containing 2 mM of L-glutamine, 10% FBS, 10 U/ml of IL-2, 1 mM of pyruvate and 1% penicillin-streptomycin. MDA-MB-231, a breast carcinoma cell line ex-

pressing only EGFR, was acquired from ATCC and maintained following the instructions.

RT nested-PCR. Total RNA was extracted with the “Ultraspec RNA isolation Kit” (Biotech) according to the manufacturer’s instructions. RNA was quantified spectrophotometrically at 260 nm and stored at -80°C . Synthesis of cDNA was performed with “Omniscript kit” (Quiagen), according to supplied protocol, using random hexamers (Pd(N)_6) and 5 μg of total RNA.

The quality of synthesis was verified amplifying a sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (GenBank BC029618). A 171 bp sequence was amplified using primers 5’-TCG CCA GCC GAG CCA CAT CG-3’ (forward) and 5’-GAA CAT GTA AAC CAT GTA G-3’ (reverse). PCR was done with 2 μl of cDNA, and 35 cycles with 56°C of annealing temperature. Reaction products (7.5 μl) were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Nested PCR was designed adapting conditions already described [20–21]. In both PCRs a *Taq*DNA polymerase Kit with Q-solution (Quiagen) and dUTP in substitution for dTTP were used. In the first round PCR the forward primer was 5’-GTA TTG ATC GGG AGA GCC G-3’ and the reverse primer was 5’-GTG GAG ATC GCC ACT GAT G-3’. PCR was accomplished in a 50 μl reaction mixture containing 8 μl of cDNA, buffer 1x, Q-solution 1x, 200 μM of dNTPs (dATP, dGTP, dCTP), 600 μM of dUTP, 0.5 μM of each primer, 2.5 mM of MgCl_2 , 3 units of *Taq*DNA polymerase and 1 unit of N-uracyl glycosylase (UNG) heat labile. Samples were maintained for 5 min at 25°C for UNG reaction and then kept for 2 min at 95°C for UNG inactivation. PCR consisted in 40 cycles in the following conditions: 95°C for 45 seconds, 60°C for 45 seconds and 72°C for 90 seconds. PCR samples were then preserved at 4°C . For the second PCR, 5 μl of the first PCR were used. The forward primer was 5’-GCG ATG CGA CCC TCC GGG-3’ and the reverse primer was 5’-TCC GTT ACA CAC TTT GCG-3’. Master mix composition was the same except for UNG. For amplification, one cycle of 94°C for 2 minutes was followed by 35 cycles of 45 seconds at 95°C , 1 minute at 55°C and 1 minute at 72°C . PCR reaction products (15 μl) were resolved on a 1.5% agarose gel stained with ethidium bromide.

The second PCR allows the amplification of 219 bp sequence from EGFRvIII mRNA (GenBank NM_201283) with a sensitivity of $1/10^7$ (sensitivity tested with dilutions of total RNA from U87MGEGFR cell line in total RNA from CTLL-2 cell line). The RT nested-PCR also allows the amplification of a 1020 bp sequence from EGFR mRNA, with a sensitivity of $1/10^6$ (sensitivity tested with dilutions of total RNA from MDA-MB-231 cell line in total RNA from CTLL-2 cell line). When both transcripts are present, the sensitivity for EGFRvIII mRNA is maintained, but for EGFR mRNA, the sensitivity is only of $1/10^4$ (preferential amplification of the shorter sequence). In each set of RT-PCRs the desired sensitivity was confirmed using dilutions ($1/10^4$ to

$1/10^7$) of total RNA from EGFRvIII expressing cell line in total RNA from CTLL-2 cell line. Whenever the sensitivity achieved was inferior to $1/10^7$, PCRs were repeated. At least two negative water blanks were included in each reaction. One of the negative water blanks of each reaction was used in the following procedures, from the cDNA to the second PCR, to assure the absence of contamination. Patients' samples were tested in triplicate.

The specificity of the RT-PCR products was confirmed by DNA sequencing the products of the second PCR obtained from expressing cell-lines and from some random selected positive blood samples.

Measurement of E-selectin and VCAM-1 concentrations. After thawing plasma samples, the levels of VCAM-1 and E-selectin were determined by commercially available ELISA kits and standards (R&D Systems), according to manufacturer's instructions. Concentrations were measured in duplicate and the average value was used for data analysis. The intra-assay variability for E-selectin and VCAM-1 was 4.8% and 3.1%, respectively. The interassay variability for E-selectin and VCAM-1 assays was 7.3% and 7%, respectively.

Statistical analysis. Differences in nonparametric data were compared with chi-square test and with Student's t test for continuous variables. A 95% confidence interval was calculated for averages values. A p -value <0.05 was considered statistically significant.

Results

In patient blood samples, the RT nested-PCR only revealed positive results at the second PCR, and only the EGFRvIII mRNA was identified (Fig. 1). The mutant transcript was detected in 45.5% of patients from group A and in 61.5% of patients from group B. There were no positive results in the 40 patients from group C (Tab. 1). The mutant mRNA was not detected neither in blood samples from 40 healthy women (results not shown), nor from RNA obtained from the CTLL-2 cell line or from the MDA-MB-231 cell line.

E-selectin plasma concentrations increased with the extension of the disease (Tab. 1). The difference was statistically significant ($p<0.025$) when comparing all patients from

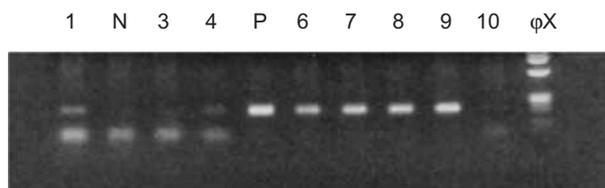


Figure 1. Results of the nested RT-PCR with patients blood samples: N – negative water-blank control; lanes 3 and 10 are negative results; P – E GFRvIII expressing cell-line; ϕ – ϕ 174-Hae III DNA ladder. The lower band corresponds to excess of primers and is only apparent when amplification is absent or minimum.

group B (26.6 ng/ml) with group C (18.8 ng/ml). The presence of circulating tumor cells was also associated with increased plasma levels of E-selectin but the difference was not significant within each group of patients. We could only detect significant statistical differences when the mean concentration from group B patients with EGFRvIII mRNA detection (27.9 ng/ml) and the mean concentration of all patients with EGFRvIII mRNA detection (26.1 ng/ml) were compared with the mean concentration from group C (18.8 ng/ml) ($p<0.025$).

For VCAM-1 the results were similar (Tab. 2). Plasma levels were also directly correlated with disease extension and increased with the identification of EGFRvIII mRNA in peripheral blood. The differences between the mean concentrations were statistically significant only ($p<0.025$) only when group C (156.9 ng/ml) was compared with all patients from group B (225 ng/ml), with patients from group B with EGFRvIII mRNA detection (231.3 ng/ml) or with all patients with EGFRvIII mRNA detection (212.55 ng/ml). Differences between groups A and B and within each group were not significant.

Discussion

In breast cancer a correct evaluation of disease extension and dissemination is essential to decide whether to use chemotherapy. Disease progression can be more accurately assessed by new molecular parameters. Even CA 15.3, the current biochemical marker available, is not enough sensitive and gives false negative results. In the last decade, cellular markers for the diagnosis of occult disseminated disease and new biochemical markers related to cancer cell proliferation, angiogenesis and invasion have been intensively investigated.

The rates of EGFRvIII positive results obtained with the RT nested-PCR are comparable to those described for other markers in peripheral blood, using nested-PCR [13, 15, 22] or real time PCR [23]. The identification of EGFRvIII mRNA in different stages of the disease reveals that the mutant expression is stable during cancer evolution, an important feature for a tumor marker.

The presence of circulating cancer cells and plasma levels of the adhesion molecules VCAM-1 and E-selectin were shown to correlate with each other and with breast cancer cell load. The rate of EGFRvIII transcript detection in peripheral blood and plasma levels of adhesion molecules increased from the group of follow-up women with treated disease (group C), to group A, consisting in women with untreated nonmetastatic cancer and, even more, to group B, including metastatic patients ($p<0.025$ when comparing groups C and B). As expected, for each group, the presence of circulating cancer cells was associated with increased levels of VCAM-1 and E-selectin, reflecting the role of these adhesion molecules in neoangiogenesis, cancer cell trafficking and invasion. In group B, all except four women, had already been

Table 1. Plasma concentrations of E-selectin in the different groups of patients

Groups		N ^o	E-selectin plasma concentration (ng/ml)		
			Average	SD	95% CI
Group A (•stages II/III)	EGFRvIII-P	5	23.1	7.5	12.67 – 33.53
	EGFRvIII-N	6	20.8	7.6	12.08 – 29.5
	Total	11	21.8	7.3	16.65 – 26.95
Group B (•stage IV)	EGFRvIII-P	8	27.9*	7.2	21.49 – 34.3
	EGFRvIII-N	5	24.5	9.6	11.2 – 37.8
	Total	13	26.6*	8	21.56 – 31.64
All stages (groups A and B)	EGFRvIII-P	13	26.1*	7.4	21.44 – 30.76
	EGFRvIII-N	11	22.46	8.32	16.59 – 28.33
	Total	24	24.4	7.9	20.99 – 27.81
Group C (follow-up)	EGFRvIII-N	40	18.8*	8.5	16.05 – 20.55

*p<0.025 comparing with group C.

EGFRvIII-P – EGFRvIII mRNA detected in peripheral blood, EGFRvIII-N – EGFRvIII mRNA not detected in peripheral blood, N^o – number of patients, SD – standard deviation, CI – confidence interval.

•FIGO classification.

Table 2. Plasma concentrations of VCAM-1 in the different groups of patients

Groups		N ^o	VCAM-1 plasma concentration (ng/ml)		
			Average	SD	95% CI
Group A (•stages II/III)	EGFRvIII-P	5	186.33	87.2	65.13 – 307.53
	EGFRvIII-N	6	171.36	62.99	99.11 – 243.61
	Total	11	178.8	77.76	123.9 – 233.7
Group B (•stage IV)	EGFRvIII-P	8	231.3*	128.7	116.7 – 345.9
	EGFRvIII-N	5	210.36	117.3	47.3 – 373.4
	Total	13	225*	119.2	149.98 – 300.02
All stages (groups A and B)	EGFRvIII-P	13	212.55*	114.13	140.75 – 284.35
	EGFRvIII-N	11	185.99	81.28	129.35 – 242.63
	Total	24	201.92	100.8	158.45 – 245.39
Group C (Follow-up)	EGFRvIII-N	40	156.9*	65.1	135.84 – 177.96

*p<0.025 comparing with group C.

EGFRvIII-P – EGFRvIII mRNA detected in peripheral blood, EGFRvIII-N – EGFRvIII mRNA not detected in peripheral blood, N^o – number of patients, SD – standard deviation, CI – confidence interval.

•FIGO classification.

submitted to chemotherapy, so, the detection of EGFRvIII transcript in the peripheral blood meant therapeutic failure.

Results also suggest that the concentrations of the adhesion molecules studied are more dependent on disease extension than on the presence of circulating cancer cells. In patients from group A, E-selectin and VCAM-1 mean concentrations were higher than in group C, although without statistical significance. Within each group, differences in concentrations between patients with or without EGFRvIII transcript detection in peripheral blood also did not reach statistical significance. VCAM-1 and E-selectin higher concentrations were reached in metastatic patients with positive results for EGFRvIII transcript detection.

Although data reveal statistically significant differences when, for instances, we compared VCAM-1 and E-selectin

mean concentrations between patients in follow-up (group C) and patients with metastatic disease and with all patients with blood identification of EGFRvIII mRNA, the confidence interval (CI) is very large (Tab. 1, 2). This high dispersion of concentration values is common even in other studies [1–10] and may raise difficulties in establishing a normal cut-off value. Apart from technical problems, the phenomenon can be explained by the great variety of situations known to interfere with the concentrations of soluble adhesion molecules, like cardiovascular disease [24], chronic renal failure [25], systemic infections [26], chronic inflammatory syndromes [27] or cigarette smoking [28]. VCAM-1 serum levels also appear to change in cancer patients undergoing peripheral blood progenitor cell (PBPC) mobilization regimens [29].

In breast cancer, the association between staging and increasing levels of E-selectin or VCAM-1 have repeatedly been described [1–10]. Serum levels of VCAM-1 have been shown to closely correlate with microvessel density in breast cancer specimens making this adhesion molecule a candidate marker of neoangiogenesis with promising applications in the evaluation of antiangiogenic therapy [2]. Elevated serum levels of E-selectin have been correlated not only with higher staging and metastasis but also with more subtle markers of aggressive biological tumor profile, like absence of oestrogen receptors or poor differentiation [4–6, 9, 10]. Its role in tumor neoangiogenesis still remains controversial [2, 5, 7, 12].

The relationship between concentrations of soluble forms of adhesion molecules and the presence of circulating tumor cells, to the best of our knowledge, has never been established

before. The results obtained, revealed that circulating cancer cells were associated with higher plasma levels of VCAM-1 and E-selectin, not only in cases with widespread disease, as in patients with clinical metastasis, but also when occult systemic disease is present, as in patients from group A. It would be interesting to verify if this phenomenon is common to other markers proposed for the diagnosis of occult systemic disease.

It is well known that the diagnosis of occult cancer cells dissemination by the existing methodologies is not always associated with the occurrence of metastasis; both false negatives and false positives exist. The association with the assessment of circulating levels of adhesion molecules, as markers of angiogenesis, may contribute to the accuracy of prognostic evaluation. This should be particularly important

for stage I patients and may have a relevant impact on treatment planning and surveillance. Future work should determine if the evaluation of individual concentration profile over time is more reliable than the comparison with a cut-off established value.

The search for tumor gene expression profiles that may predict future metastasis is a difficult task, as revealed by the lack of reproducibility between results of different groups [30]. Neoangiogenesis is a crucial step in tumor growth and dissemination. Tumor microvessel density and VEGF-family expression have been correlated with poor prognosis and short survival, but conclusions are not unequivocal [31, 32]. Increased levels of circulating endothelial cells and their immature precursors are also associated with tumor neoangiogenesis and metastasis. Different methodologies have been applied to quantify these cells, like flow cytometry, real-time PCR [34] or immunostaining [35]. The evaluation of the levels of circulating adhesion molecules by ELISA is technically less complex and easier to perform. Further studies are needed to establish and compare the predictive values of these different approaches of assessment of tumor angiogenesis. The research of the last decades suggests that, most probably, no single method or marker will fully predict cancer outcome and efforts have to be conciliated to select the most clinically useful approaches.

Besides being important as prognostic factors, markers of angiogenesis may also constitute new targets for cancer therapy and contribute to evaluate and monitor the efficacy of antiangiogenic therapy.

Conclusion

Increased plasma levels of VCAM-1 and E-selectin are associated with advanced breast cancer stage and with the presence of circulating cancer cells. The combined analysis of these parameters may contribute to a more accurate evaluation of cancer dissemination.

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