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Molecular detection of EGFRvIII-positive cells in the peripheral blood of breast cancer patients

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ABSTRACT

The aim of this study is to evaluate epidermal growth factor receptor variant III, EGFRvIII, a cancer specific mutant, as a possible marker for the diagnosis of breast cancer occult systemic disease. EGFRvIII mRNA was identified by an RT-nested PCR with a high sensitivity. In 102 women studied, the mutant was detected in the peripheral blood of 30% of 33 low risk, early stage patients, in 56% of 18 patients selected for neoadjuvant chemotherapy, in 63.6% of 11 patients with disseminated disease and 0% of 40 control women. In low risk, early stage patients, the presence of one or more tumour characteristics predicting recurrence such as the absence of oestrogen receptors and the presence of ERBB2 or histologic grades G2/G3 was significantly associated with EFGRvIII detection ($p < 0.05$). EGFRvIII mRNA has characteristics to be a useful marker for the diagnosis of occult systemic disease in breast cancer. Follow-up studies will evaluate its clinical value as a decision criterion for systemic therapy.

1. Introduction

Breast cancer is the second leading cause of death by cancer in most Western countries. Prognosis is mainly dependent on the extension of the disease, but even with the most recent medical advances, 20–30% of patients with an apparently localised cancer will relapse. The most likely reason is an early, occult systemic dissemination.

The diagnosis of occult systemic disease may constitute a significant improvement in the treatment and prognosis of breast cancer.[1–3] It may help to improve staging, especially in patients with apparently localised disease, to monitor the effectiveness of therapy and to allow a pre-clinical diagnosis of recurrences in patients already in follow-up.

PCR methodologies have been widely used in the detection of occult systemic disease in blood and bone marrow of cancer patients. However, no suitable marker is available. Tumours are very heterogeneous and may express different mRNAs and proteins. Unfortunately, the most frequently and highly expressed markers are not cancer specific and false positives from illegitimate transcription are common in PCR-based technologies. Therefore, the search for specific markers remains an important issue to pursue.

Epidermal growth factor receptor variant III (EGFRvIII) is the most common mutant of EGFR, a receptor described in all cell types with the exception of haematopoietic cells. The mutant results from an in-frame deletion of the coding exons 2–7, corresponding to a deletion of 801 bp from the mRNA.[4–6] The rearrangement creates a new peptide sequence at the extracellular domain, with a new amino acid, glycine, at the fusion junction. EGFRvIII may result from genomic deletion associated with EGFR amplification, or from alternative splicing.[7–9]

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EGFRvIII is a ligand independent and constitutively active receptor.[8,10] Its expression has been detected in a wide variety of human malignancies, including up to 50–78% of breast cancers.[8,11–13] No detectable level of protein or mRNA of EGFRvIII has, however, been observed in normal adult tissues, including normal breast tissues.[7,11,14] There is compelling evidence that the expression of EGFRvIII enhances tumourigenicity[10,15,16] and invasiveness[10,16,17] and is associated with chemo and radioresistance.[18,19] Efforts are being developed to delineate cancer specific therapies having EGFRvIII as a target.[20,21]

In this work, EGFRvIII mRNA is evaluated as a possible marker for the diagnosis of breast cancer occult systemic disease.

2. Materials and methods

2.1. Cell lines

U87MGΔEGFR, 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 μg/ml of Geneticin. CTLL-2, a lymphoblastic cell line, was a generous gift from the Histocompatibility Centre 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 expressing only EGFR, was acquired from ATCC and maintained following the instructions.

Table 1 – Characteristics and results of the different groups

Population characteristics	Total population	EGFRvIII positives
Group A (Age: mean – 63.6 ± 11.3)		
T2N0	9	3
T1N0	24	7
Total	33	10 (30%)[a]
Group B (Age: mean – 48 ± 12.59)		
IIA[b]	4	2
IIB[b]	3	2
IIIA[b]	4	3
IIIB[b]	7	3
Total	18	10 (56%)[a]
Group C (stage IV)[b] (Age: mean – 56.7 ± 17.6)		
Bone metastasis	6	4
Multiple sites[c]	4	3
Other[d]	1	0
Total	11	7 (63.6%)[a]
Group D (Age: mean – 41.3 ± 11.4)		
Total	40	0

a $p > 0.05$ (Yates' p value).
b FIGO classification.
c With bone and visceral metastasis.
d Supra-clavicular lymph node metastases.

2.2. Patients

One hundred and two women divided into four groups were studied: group A, with 33 T1N0M0 or T2N0M0 (TMN, clinical classification) breast cancer patients, with a mean age of 63.6 ± 11.3 and with tumours of 3 cm or less of diameter, selected for curative surgery; group B with 18 high risk patients, with a mean age of 48 ± 12.6, selected for neoadjuvant chemotherapy; group C, with 11 patients with disseminated disease, with a mean age of 56.7 ± 17.6 and group D, a control group of 40 women free of any type of cancer with a mean age of 41.3 ± 11.4 (Table 1). Group B is heterogeneous and includes women from stage II A to stage III B (FIGO classification), as described in Table 1. In group A, blood was collected before surgery and in groups B and C before chemotherapy. Blood samples were processed within 1 h of collection. All patients had carcinomas of ductal invasive histological type, except one with an intraductal carcinoma with microinvasion and another with an invasive tumour with ductal and lobular differentiation. Patients were undergoing treatment in the Gynaecological Department of Coimbra University Hospital. Samples were consecutively collected during the period of 1 year according to patient consent and sample quality demands. Ethical approval was obtained from the Hospital Ethical Committee and all subjects gave their informed consent. Group D included University and Hospital staff, with a medical consultation during the last year, and women attending the Gynaecological Department for benign disorders.

2.3. Patient samples

Twenty-three tumour samples were collected from breast cancer patients submitted to surgery in the Gynaecological Department, 18 of which were from the group A patients. All these samples were of ductal invasive histological type. Samples were collected and immersed in RNA*later* (Qiagen) within 1 h after surgery and kept at −20 °C or −80 °C until RNA extraction.

The mononuclear cell fraction of 10 ml of EDTA treated blood was separated by gradient density centrifugation with Ficoll. A total of 5×10^6–1×10^7 viable cells were used for RNA extraction.

2.4. RNA extraction and RT-PCR

Total RNA was extracted from cell lines with the 'Rneasy Kit' (Quiagen), and from blood and tissues with the 'Ultrasepc RNA isolation Kit' (Biotecx) 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 the supplied protocol, using random hexamers ($Pd(N)_6$) and 5 μg of total RNA.

The quality of synthesis was verified by 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 accomplished 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 stained with ethidium bromide.

Nested PCR was designed adapting conditions already described.[11,22] In both PCRs, we used *Taq* DNA polymerase Kit with Q-solution (Quiagen) and dUTP in substitution for dTTP. 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 performed with a 50 μl reaction mixture containing 5–8 μl of cDNA, buffer 1×, Q-solution 1×, 200 μM of dNTPs (dATP, dGTP, and dCTP), 600 μM of dUTP, 0.5 μM of each primer, 2.5 mM of $MgCl_2$, 3 U of *Taq* DNA polymerase and 1 U 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 s, 60 °C for 45 s and 72 °C for 90 s. PCR samples were then preserved at 4 °C. For the second PCR, 1–5 μl of the first PCR was 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 min was followed by 35 cycles of 45 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C. 8 μl of cDNA and 5 μl of the first PCR were used in the higher RNA dilutions in sensitivity assays ($1/10^5$–$1/10^8$) and with patients and control blood samples.

For EGFRvIII, the first PCR amplifies a sequence of 352 bp and the second PCR amplifies a sequence of 219 bp (GenBank NM_201283). For EGFR, the sequences amplified in the first and second PCR are 801 bp longer. Reaction products (15 μl) from the first and second PCRs were resolved on a 1.5% agarose gel stained with ethidium bromide.

In each set of RT-PCRs, the desired sensitivity was confirmed using dilutions ($1/10^4$–$1/10^7$) of total RNA from EGFRvIII expressing cell line in total RNA from CTLL-2 cell line, as described in the sensitivity tests. Patients' samples were tested in triplicate and dilution samples in duplicate. Whenever both $1/10^7$ dilutions were negative, the sensitivity of the assay was considered to be insufficient and 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. In the first and second PCRs, the samples used to test the sensitivity were the last to be handled, from the higher to lower dilution, ending with the negative water blanks. Tissue processing, RNA extraction, RT-PCR assay set-up and post-PCR product analysis were performed in separate rooms to avoid contamination.

Sensitivity was established by serially diluting total RNA from U87MGΔEGFR or from MDA-MB-231 cell lines into 5 μg of total RNA extracted from CTLL-2 cell line. Serial dilutions were also done with a mixture of equal quantities of RNAs from EGFRvIII and EGFR expressing cell lines into 5 μg of total RNA from CTLL-2 cell-line. Dilutions ranged from 1/10 to $1/10^8$.

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 and tumour samples. The specificity was also confirmed by applying the same RT-nested PCR assay to cells that do not express EGFRvIII: MDA-MB-231 and CTLL-2 cell lines and normal nucleated blood cells.

The protocol assures that only mRNA is amplified: for EGFR, the DNA sequences corresponding to the product amplified by the RT-PCR include several introns; for EGFRvIII, the reverse primer of the second PCR spans exons 8 and 9 junction.

2.5. *Statistical analysis*

Student's *t* test for continuous variables and Yates' χ^2 test for categorical variables were used. A p-value <0.05 was considered statistically significant.

3. Results

As described in the last section, the sensitivity results concern RNA dilutions. For EGFRvIII marker, a sensitivity of $1/10^4$ was achieved in the first PCR. With nested PCR the sensitivity was of $1/10^7$. For EGFR, the first PCR had a sensitivity of $1/10^3$ and the nested PCR a sensitivity of $1/10^6$ (Fig. 1a). When both markers were simultaneously used, for higher dilutions ($1/10^5$–$1/10^7$), only the deleted form was detected, achieving the same sensitivity of $1/10^7$ (Fig. 1b). Dilutions with eight positive results out of ten were considered to be within the sensitivity threshold. For $1/10^8$ dilutions, only two in ten samples were positive, so this dilution was considered to be above the sensitivity of the assay.

Confirming the specificity of the assay and of the markers, EGFRvIII mRNA was only identified from U87MGΔEGFRF cell line and not from MDA-MB-231 or CTLL-2 cell lines or normal leukocytes. EGFR mRNA was also identified only in MDA-MB-231 cell line.

As shown in Table 1, in group D, of control women, there were no positive cases for EGFR or EGFRvIII. In the patient population, in group A, 10 were EGFRvIII positive (30%); in group B, 10 (56%) were EGFRvIII positive and in the metastatic group, 7 (63.6%) were also EGFRvIII positive (Yates' p value >0.05). The positive results were only seen with the nested PCR and not in the first PCR, even in blood samples from metastatic patients (Fig. 1c). Patients' samples were done in triplicate and all positive results were positive at least twice.

For group A, some risk factors of tumour recurrence were analysed between EGFRvIII positive or negative patients in peripheral blood (Table 2). There was no significant difference for age, microscopic diagnosed lymph node metastasis or tumour size. For other biological markers associated with tumour relapse, such as absence of oestrogen receptors (ER), G2–G3 histologic grade and expression of ERBB2, there was a clear higher incidence in patients EGFRvIII-positive in peripheral blood, reaching a statistically significant difference ($p < 0.05$) when the presence of one or more of these factors was considered.

To confirm the high frequency of breast cancers positive for EGFRvIII mRNA described in the literature, a group of 23 breast cancer samples, 18 of which belonging to group A of patients, was studied. In other patients from group A, it was not possible to collect tissue samples mostly due to the reduced dimensions of the tumours. Results showed that

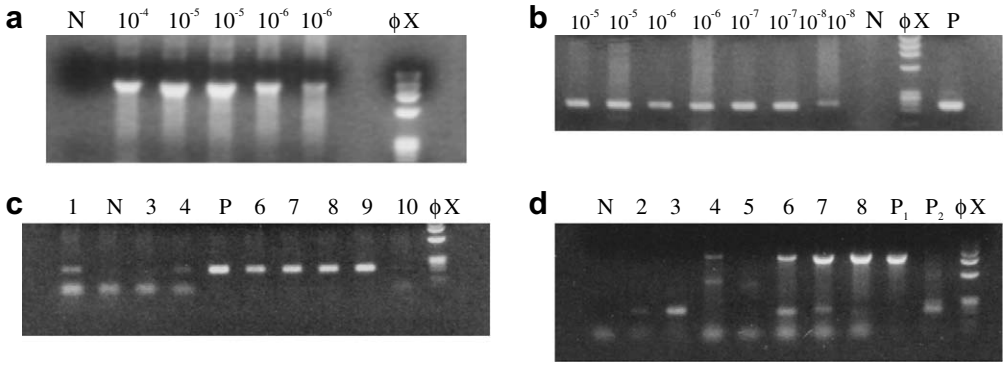

Fig. 1 – (a) Sensitivity of nested RT-PCR for EGFR: N, negative control with RNA from CTLL-2 cell line; 10^{-4}–10^{-6} – serial dilutions of RNA from EGFR-expressing cell line in RNA from the non-expressing cell line, CTLL-2; φX – φX 174-*Hae*III DNA ladder. (b) Sensitivity of nested RT-PCR for both EGFR and EGFRvIII: 10^{-5}–10^{-8} serial dilutions of RNAs from both EGFR and EGFRvIII-expressing cell lines in RNA from the non-expressing cell line; N, negative, water-blank control; φX – φX 174-*Hae*III DNA ladder; P, EGFRvIII-expressing cell line. In these higher dilutions, the sequence of EGFRvIII is preferentially amplified. (c) Results of the nested RT-PCR of patients blood samples: N, negative water-blank control; lanes 3 and 10 are negative results; P, EGFRvIII expressing cell-line; φX – φX 174-*Hae*III DNA ladder. (d) Results of the nested RT-PCR of tumour samples: N, negative water-blank control, lanes 2–8, tumour samples; P_1, EGFR expressing cell line; P_2, EGFRvIII expressing cell line; φX – φX 174-*Hae*III DNA ladder. The lower band seen in (c) and (d) corresponds to excess of primers and is only apparent when amplification is absent or minimum.

Table 2 – Recurrence predictive factors in group A patients

	Age	Tumour size ⩾2 cm	pN1	ER negative	Grade G2–G3	ERBB2 positive	ER-/G2-G3/ERBB2+	
							⩾1*	⩾2*
EGFRvIII positive, *N* = 10	58.9 ± 11.4	3/10 (30%)	1/10 (10%)	3/10 (30%)	9/10 (90%)	5/10 (50%)	10–10* (100%)	7/10 (70%)
EGFRvIII negative, *N* = 10	64.6 ± 11.2	6/23 (26%)	4/23 (17.4%)	3/23 (13%)	12/23 (52%)	9/23 (39%)	13/23* (56.5%)	9/23 (43.55%)

Statistic: Student's-*t* test for age and χ^2 test for other variables; *$p < 0.05$ for the presence of at least one of the factors; pN1, lymphatic node microscopic metastases; ER, for absence of oestrogen receptor; ERBB2+ for presence of this receptor.

82.6% of the tumours were positive for EGFRvIII and/or EGFR, 65% being positive for EGFRvIII in nested PCR (Fig. 1D). Of the 10 group A patients with EGFRvIII identified in the peripheral blood, only three had the tumour studied: two samples were positive for the mutant and one for both the mutant and the normal transcript.

4. Discussion

In this paper, EGFRvIII mRNA is evaluated as a possible marker for the diagnosis of breast cancer occult systemic disease. Characterisation of tumour samples confirmed the high frequency of the mutant in breast cancer: 82.6% of the tumours were positive for EGFRvIII and/or EGFR. The RT-nested PCR assay achieved a high sensitivity of $1/10^7$ for EGFRvIII and $1/10^6$ for EGFR. The lower sensitivity for EGFR, especially when both markers were present, was probably due to the longer amplification sequence, and explains the absence of positive results. The mutant was never detected in the first PCR, even in the metastatic group of patients, confirming the need for a highly sensitive assay. The rate of EGFRvIII positive cases in patients with advanced cancer was higher than in patients with clinically localised disease, supporting the usefulness of this assay. 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 tumour marker. Also, in the early stage patients, the presence of one or more tumour characteristics predicting recurrence such as the absence of oestrogen receptor (ER) and the presence of ERBB2 or histologic grades G2/G3 was significantly associated with EFGRvIII positive results in the peripheral blood ($p < 0.05$). This is particularly interesting because ERBB2 and G2 histologic grade are not always considered for adjuvant chemotherapy prescription. Though the number of patients does not allow definitive conclusions, results suggest that these two tumour phenotypic characteristics and EGFRvIII positive results in peripheral blood might help to identify those patients with T1N0M0 disease, not submitted to chemotherapy, but that still will relapse. Giving that in these early stage patients, the incidence of recurrences and of blood detection of markers of occult systemic disease is usually low, follow-up studies with larger number of patients must be accomplished to confirm the usefulness of EGFRvIII.

The lack of association between positive results in the peripheral blood and tumour size or lymph-node involvement has been described in other studies.[1,2] There are evidences that distinct biological processes support lymph-node and haematic dissemination.[23]

The rates of positive results obtained with the RT-nested PCR are comparable to those described by other authors in peripheral blood, using nested PCR,[2,3,24] real time PCR[25] or recently developed antibody-based assays.[26] In sensitivity tests, for practical purposes, mRNA dilutions were used, as sensitivity was tested in every PCR set of blood samples. Though the dilution of cells from expressing cell lines in blood samples is certainly a more desirable method, RNA and even cDNA dilutions have often been used.[24,26–28] To avoid contamination of blood samples from cell epidermis some authors proposed to discard the first millilitres of blood, but recently, this cause of false positive results was questioned.[25] Therefore, and because the EGFRvIII is a cancer specific marker, we used the whole blood sample in patients and controls.

EGFR has already been described as a good marker for the detection of circulating cancer cells in breast cancer patients,[24,26] though false positives may appear because of illegitimate transcription. As a marker, EGFRvIII offers the advantage of being cancer specific, thus, reducing false positives.

A double-mutant EGFRvIII, with an additional deletion of exons 12 and 13, has rarely been described[29] and may be identified by our protocol. This rare mutant has only been associated with EGFR positive tumours, so a positive result in peripheral blood would have the same meaning as an EGFRvIII positive result.

As a quantitative value for positive results may be important in prognosis evaluation, a real-time PCR with *TaqMan* probes for EGFRvIII and EGFR was tried, but the sensitivity achieved, of $1/10^4$, was insufficient, and, as expected by the nested-PCR results, no positive cases were detected in blood samples. In the diagnosis of occult systemic disease, real time PCR has mostly been used in patients with advanced breast cancer[3,25] and few authors have described sensitivities higher than $1/10^5$ in this specific application of real-time PCR.[25] Although real-time PCR is a highly reproducible quantitative PCR, contrarily to image-assisted systems, it cannot accurately quantify the number of tumour cells corresponding to the mRNA levels or evaluate the extension of the disease.[25,28] For the diagnosis of occult systemic disease in solid tumours, this methodology has mainly been used to eliminate the background signal obtained from almost all markers, by establishing a cutoff point that distinguishes true positives from false positives.[3,25,28,30]

The complexity of metastasis explains its unpredictability and enhances the need for highly sensitive and specific methodologies enabling the identification of specific markers implicated in tumourigenesis and metastasis. Unfortunately, specific markers, like EGFRvIII or mammaglobin[31], demand assays with sensitivities not easily achieved.

Although the expression of EGFRvIII has been described in 50–72% of breast cancers by many authors,[8,11–13] using different methodologies (PCR, immunohistochemistry and Western blotting), including laser capture technology,[13] recently the absence of EGFRvIII expression in breast cancer paraffin-embedded samples was reported.[32] These conflicting results may be explained by differences in sensitivity assays and in sample management. So that archived tissues accurately reflect mRNA expression before fixation, some factors have to be optimised, namely time from surgical excision to fixation.[33] This is critical when concerning breast cancer samples, because of the pathological procedures necessary for staging. Less abundant mRNAs may be lost. It was also proven that the process of formalin fixation and paraffin embedding differentially affects the ability to analyse different mRNA species even from the same gene.[33]

Despite the frequent expression of EGFRvIII in human epithelial cancers, it has been difficult to detect EGFRvIII expression in most human cancer cell lines.[18,32] The common explanation, either a growth disadvantage *in vitro* or a positive selection for EGFRvIII *in vivo*, was recently confirmed by two different groups.[21,34] A similar phenomenon has been verified with other genes.[35]

In the diagnosis of breast cancer occult systemic disease, it is important to search for a panel of specific cancer markers to create personalised protocols. EGFRvIII has characteristics to be an interesting candidate, though it requires a highly sensitive methodology. It has the advantage of being a cancer exclusive mutation associated with tumourigenicity and metastasis and of being a good target for specific therapy. In early stage disease, the detection of EGFRvIII in peripheral blood is associated with indicators of tumour aggressiveness. Larger sample studies and long term observation will evaluate its clinical value as a decision criterion for systemic therapy.

Conflict of interest statement

None declared.

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