Abstract. It is now widely recognized that translation factors are involved in cancer development and that components of the translation machinery that are deregulated in cancer cells may become targets for cancer therapy. The eukaryotic Release Factor 3 (eRF3) is a GTPase that associates with eRF1 in a complex that mediates translation termination. eRF3a/GSPT1 first exon contains a (GGC)n expansion coding for proteins with different N-terminal extremities. Herein we show that the longer allele (12-GGC) is present in 5.1% (7/137) of the breast cancer patients analysed and is absent in the control population (0/135), corresponding to an increased risk for cancer development, as revealed by Odds Ratio analysis. mRNA quantification suggests that patients with the 12-GGC allele overexpress eRF3a/GSPT1 in tumor tissues relative to the normal adjacent tissues. However, using an in vivo assay for translation termination in HEK293 cells, we do not detect any difference in the activity of the eRF3a proteins encoded by the various eRF3a/GSPT1 alleles. Although the connection between the presence of eRF3a/GSPT1 12-GGC allele and tumorigenesis is still unknown, our data suggest that the presence of the 12-GGC allele provides a potential novel risk marker for various types of cancer.

Introduction

There is presently a growing body of evidence that supports the involvement of translation in cell proliferation and cancer development (1-3). This includes overexpression of initiation (4), elongation (5), and more recently, termination factors (6). Eukaryotic translation termination is governed by two release factors, eRF1 and eRF3, that associate in a complex which binds to the ribosomal A site. eRF1 recognises the three stop codons, and promotes the release of the nascent polypeptide chain. eRF3 is a small GTPase that enhances eRF1 activity (7). The C-terminal region of eRF3 proteins is highly conserved and essential for translation termination and interaction with eRF1 whereas the N-terminal region varies in both length and sequence among species. Although this domain has been reported to be dispensable for translation termination (7,8), it was recently shown that it influences eRF3 functions in translation and possibly in other cellular processes (9,10).

In humans, eRF3 has two distinct isoforms, eRF3a encoded by eRF3a/GSPT1 gene located in 16p13.1 (11) and eRF3b, encoded by eRF3b/GSPT2 gene located in Xp11.21-23 (12). eRF3a mRNA is abundant in all tissues and its level varies during the cell cycle, whereas eRF3b mRNA is poorly expressed in most mouse tissues tested, except in brain (13). It was previously reported that eRF3a/GSPT1 mRNA level is increased in 70% of the intestinal type gastric tumors (6) and strongly decreased during human chondrocyte differentiation (14).

eRF3a and eRF3b proteins share 87% identity, most of the differences being concentrated in their N-terminal domains. The N-terminal domain of human eRF3a contains a poly-glycine expansion encoded by a stable (GGC)n expansion in eRF3a/GSPT1 first exon. There are five known alleles, which encode 7, 9, 10, 11 and 12 glycines. Our group has reported a strong association between the longest allele (12-GGC) and gastric cancer development (15), which was shown to be a germline mutation. Because of the absence
of the 12-GGC allele in healthy control population, it was suggested that the presence of eRF3a/GSPT1 12-GGC allele was directly associated with gastric cancer development.

In the present study, we aimed to evaluate eRF3a/GSPT1 as a susceptibility locus for cancer development, searching for further evidence in other cancer models. Breast cancer was chosen because it is one of the most common and serious malignancies affecting women. In addition, more than 75% of the hereditary tumors result from inherited defects in genes that are still to be identified (16).

Breast cancer patients were genotyped for the (GGC)n polymorphism in order to determine the frequency of the 12-GGC allele and the associated cancer risk. We also investigated the correlations between the different GGC alleles and the pattern of eRF3a/GSPT1 expression in tumor samples. In addition, we looked for correlations between clinical/morphological parameters, the eRF3a/GSPT1 genotype and eRF3a/GSPT1 gene expression levels. Finally, a functional in vivo assay was used to search for differences in the translation termination activity of eRF3a proteins encoded by the different GGC alleles.

Material and methods

Patients and sample collection. We analysed 137 breast tumors collected from surgical resections in Fernando da Fonseca Hospital, after informed consent. This study was approved by the Scientific Committee of the hospital. The tumors were diagnosed as breast cancers by histopathological examination performed in the department of Surgical Pathology. A sample of 135 healthy blood donors with matched demographic characteristics was used as control population.

Freshly frozen samples from 52 tumors, together with a sample from the adjacent non-neoplastic tissue, were collected for gene expression analysis. Those tissue samples were stored in RNAlater (Ambion, Austin, TX, USA) at -20°C immediately after surgery until RNA extraction. Data were stored in RNAlater (Ambion, Austin, TX, USA) at -20°C until RNA extraction. DNA extraction, RNA extraction, and cDNA synthesis. DNA was extracted either from two 7 mm thick formalin fixed, paraffin wax embedded tissue sections using chelex resins (17) or from total blood using the DNeasy tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

RNA was extracted from fresh tissues using the SV Total RNA Isolation System (Promega, Mannheim, Germany), as recommended by the manufacturer. First strand cDNA was synthesised using MultiScribe™ reverse transcriptase (Applied BioSystems, Foster City, CA, USA), with random hexamers, according to the manufacturer's instructions, in a total volume of 50 ml; samples were incubated for 10 min at 25°C, 30 min at 48°C, and finally for 5 min at 95°C.

Patients genotyping. A fragment of eRF3a/GSPT1 exon 1 including the (GGC)n polymorphism was PCR amplified, from patient's genomic DNA, using a 6'-FAM labelled forward primer and a non-labelled reverse primer as previously described (15). Fragment sizes were detected in an ABI PRIM 3700 DNA Analyzer sequencer and analysed with GeneScan 3.7. software (ABI).

Relative gene expression. Expression of the eukaryotic translation factors eRF3a, eRF3b, and eRF1 in tumor samples relative to normal adjacent tissues was investigated using real-time quantitative RT-PCR using gene-specific primers and TaqMan probes. All the samples were analysed in triplicate as described by Malta-Vacas et al (5).

Relative quantitative of the miRNA levels of the target genes (quantity of transcripts of the target in tumor samples relative to normal tissues) was determined using the ΔΔCT method (18). Briefly, the amount of target was normalised to the endogenous reference gene (18S rRNA) and its expression in tumor samples was calculated relative to a calibrator (normal adjacent sample). Final results are expressed as N-fold difference in tumor expression relative to non-neoplastic adjacent tissue.

Plasmid constructions and cell line

Plasmids. Plasmid expressing small interfering RNA si-3a1 targeting eRF3a mRNA and plasmid pCMV-heRF3a expressing human eRF3a have been previously described (19).

The plasmid pCMV-heRF3a was used to construct the plasmids expressing the different GGC allele of eRF3a, eRF3a/GSPT1 exon 1 fragments with GGC repeat length of 7, 9, 10, 11, and 12 were amplified by PCR from genomic DNA of genotyped patients, using 5'-CCATTTCCTCGCTCTCT GTCACC-3' and 5'-ACTCAACGTCAACGCCAG-3' primers. The PCR fragments were digested with Xmal and BamHI, purified, and ligated into the Xmal/BamHI fragment of pCMV-heRF3a. The five eRF3a expression plasmids
obtained were sequenced to verify the correct sequence and GGC repeat length of the inserts.

**Cell culture and electroporation.** The 559C cell line is a derivative of human HEK293 cell line stably expressing a lacZ gene in which the coding sequence is interrupted by a TAG stop codon. Cell cultures and electroporation was performed as previously described (19). Briefly, the 559C cells were depleted in eRF3a by electroporation of a plasmid expressing a siRNA (si-3a1) directed against eRF3a mRNA or pSuper as a control and re-electroporated 3 days later with either the empty vector pBK-CMV (3a1/pBK and sup/pBK) or plasmid expressing the eRF3a variants (3a1/7GGC, 3a1/10GGC, 3a1/11GGC, and 3a1/12GGC).

The antibodies against human eRF3a and eRF1 were previously described (19). The anti-α-tubulin (DM1A), and anti-rabbit IgG and anti-mouse IgG peroxidase-linked antibodies were from Amersham Biosciences. Western blotting were performed as previously described (19).

**Readthrough assay.** The pellets of electroporated cells were lysed by freeze-thawing cycles and cell lysates were assayed for total proteins and β-galactosidase activity. For each sample, total protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce). The β-galactosidase assays were performed using the Luminescent betagalactosidase Detection Kit II (Clontech), following the manufacturer's instructions. The β-galactosidase activity was measured as relative light units (RLU) using a single photon count program on scintillation counter. The β-galactosidase activity as expressed as RLU/mg of total proteins and the readthrough efficiencies were calculated as sample activity relative to negative control activity (cell extract 3a1/pBK). Thus, for each experiment, the percent readthrough level relative to readthrough level of cells electroporated with plasmid expressing si-3a1 and re-electroporated with pBK-CMV (3a1/pBK) was calculated and expressed as readthrough efficiency.

**Statistical analysis.** Allele frequencies and Fisher's tests were calculated in GENEPOP (version 1.2) (20). Odds ratios were calculated according to (21). All other statistical analysis were performed in SPSS 14.0 for Windows. Spearman's Rho coefficient was used to investigate correlations between the patient's genotypes and gene expression levels, and also between both genetic parameters and clinical parameters. In all cases, the tests were considered significant when the p-values were <0.05.

**Results**

*eRF3a/GSPT1* GGCn alleles in breast cancer patients. The genotypes of 137 breast cancer patients and 135 healthy blood donors were determined by fluorescent PCR amplification and peak detection in an automatic sequencer. For each patient, normal and tumor tissue samples were analysed and no discrepancies were detected. Therefore, the allele length variations were considered germline mutations and validated for comparison with the frequencies obtained from blood samples analysis for the control group.

The five known *eRF3a/GSPT1* GGC alleles corresponding to 7, 9, 10, 11 and 12 GGC repeat units were identified. The 10-GGC allele was the most frequent in both groups (Fig. 1A). Note that repetitive sequencing revealed that the 8-GGC allele (15) carries only 7 GGC repeats and was renamed 7-GGC.

Using the Fisher's test, we detected significant differences between the breast cancer and control populations in genotypic frequencies (χ²= 6.618; p=0.03655) but not in allelic frequencies (χ²=5.535; p=0.06282). The 12-GGC allele, which was only detected in cancer patients (allelic frequency=0.025), was associated with an 11-fold increase in breast cancer risk (OR=11.32; CI=0.64-199.93). Regarding genotypes, the 12-GGC allele was presented in two different genotypes, 10-12GGC and 11-12GGC (Fig. 1B), which were associated with 6.26- and 4.87-fold increased risk for breast cancer, respectively.

*eRF3a/GSPT1* mRNA expression in tumor tissues. Relative expression of *eRF3a/GSPT1* gene was determined by real-time RT-PCR using gene specific primers and TaqMan probes in 52 breast cancer patients. *eRF3a/GSPT1* was classified as overexpressed when its mRNA level in tumor tissue samples was at least the double of that in adjacent non-neoplastic tissues. Underexpression was defined as less than half the expression level relative to adjacent non-neoplastic samples. The results showed that *eRF3a/GSPT1* was overexpressed in 44% (23/52) of the tumor samples whereas it was under-expressed in 20% (10/52) of the samples. Moreover, in 17% (9/52) of the tumors the expression level of *eRF3a/GSPT1* was 5.5-fold higher than in non-neoplastic adjacent tissue.

To explore if different *eRF3a/GSPT1* expression levels corresponded to different tumor types, a subset of 27 invasive ductal carcinomas (IDC) was analysed, but the same expression pattern was observed – 44% (12/27) of the tumors overexpressed *eRF3a/GSPT1* (Fig. 2A). This suggests that the variations of *eRF3a/GSPT1* mRNA expression are not specific for a particular type of tumor.

*eRF3b/GSPT2* mRNA levels were also quantified to determine whether the changes in *eRF3a/GSPT1* levels were balanced by modifications in *eRF3b/GSPT2* gene expression. We observed that *eRF3b/GSPT2* mRNA was undetectable in normal tissue samples and only poorly expressed in 25% (7/27) of the tumor tissues. There was no correlation between *eRF3a/GSPT1* and *eRF3b/GSPT2* expression levels.

Because eRF1 is the partner of eRF3 in the translation termination complex, we also examine the expression status of eRF1 mRNA. eRF1 mRNA was overexpressed in 33% of the tumor samples, underexpressed in 22% and we did not detect any variation between normal and tumor tissues in 44% (12/27) of the cases analysed (Fig. 2B). However, no significant correlation was found between the variations of eRF1 and eRF3a/GSPT1 mRNA levels (Fig. 2).

In addition, we examined a set of biomarkers that are currently used to determine the prognosis and treatment of breast cancer, i.e., c-erb-B2, ER and PR proteins expression (22), and the status of different clinical parameters, grade of differentiation, tumor size, node status, vascular invasion, surgical margins and previous chemo/radiotherapy treatment (data not shown). We did not detect any significant correlation.
between these parameters and the variations of eRF3a/GSPT1 mRNA levels.

Allele-specific expression of eRF3a/GSPT1 mRNA. In order to determine whether the GGC repeat length influence eRF3a/GSPT1 expression or whether variations of eRF3a/GSPT1 expression were linked to a specific GGC allele, we examined the genotype of the 52 patients for whom the expression status of eRF3a/GSPT1 in tumor tissues has been determined. We failed to find significant correlations between the GGC repeat length and the levels of eRF3a/GSPT1 transcripts in breast cancer patients, when the 52 patients analysed were pooled (Table I). However, almost 60% of the patients with the 7-10GGC genotype revealed underexpression of eRF3a/GSPT1. Regarding the subset of the 27 patients with IDC type tumors, all 3 patients carrying the 7-GGC allele underexpressed eRF3a/GSPT1 mRNA in tumor samples (samples identified with * in Fig. 2A). Moreover, these tumor tissues were positive for c-erb-B2 immunoexpression. At the opposite, the two 12-GGC alleles...
detected within the 52 cases of breast cancer were within the 27 cases of IDC and both overexpressed the eRF3a/GSPT1 and eRF1 mRNAs (samples identified with # in Fig. 2A). Interestingly, both tumors were positive for RE and RP immunoexpression.

Translation termination activity of eRF3a forms encoded by the GGCn alleles. We used complementation experiments in eRF3a-depleted cells to analyze the translation termination activity of the 5 eRF3a variants encoded by the 5 known alleles of eRF3a/GSPT1 gene. Parallel cultures of 559C cells, a cell line expressing a lacZ reporter gene interrupted by a premature stop codon, were depleted in eRF3a by electroporation of a plasmid expressing a siRNA, si-3a1, specifically targeting eRF3a/GSPT1 mRNA. Three days after electroporation, cells were re-electroporated with the plasmids expressing the eRF3a variants. Three days later, the effect on stop codon readthrough was analyzed by measuring the β-galactosidase activity. Cells electroporated with the empty vector pSuper and re-electroporated with plasmid pBK-CMV served as a negative control (Fig. 3A, lane sup/pBK). Cells electroporated with plasmid expressing si-3a1 and re-electroporated with plasmid pBK-CMV served as standard for readthrough level promoted by eRF3a depletion (Fig. 3A, lane 3a1/pBK). For each electroporation experiment, the amount of endogenous eRF1 and eRF3a overexpressed proteins was verified by Western blot analysis. As shown

Table I. eRF3a/GSPT1 gene expression levels in 52 breast cancer patients.

<table>
<thead>
<tr>
<th>Alleles (GGC)n</th>
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<th>OE</th>
<th>NV</th>
<th>UE</th>
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<tbody>
<tr>
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<td>30</td>
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<td>100</td>
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<table>
<thead>
<tr>
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<th>OE</th>
<th>NV</th>
<th>UE</th>
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<td>0</td>
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<td>0</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>1-10</td>
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<td>34.6</td>
<td>19.2</td>
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<td>53.9</td>
<td>7.7</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>2</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>11-12</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Variations in the expression level are analysed for each allele and genotype. OE, overexpression; NV, no-variation; UE, underexpression.

Translation termination activity of eRF3a forms encoded by the GGCn alleles. We used complementation experiments in eRF3a-depleted cells to analyze the translation termination activity of the 5 eRF3a variants encoded by the 5 known alleles of eRF3a/GSPT1 gene. Parallel cultures of 559C cells, a cell line expressing a lacZ reporter gene interrupted by a premature stop codon, were depleted in eRF3a by electroporation of a plasmid expressing a siRNA, si-3a1, specifically targeting eRF3a/GSPT1 mRNA. Three days after electroporation, cells were re-electroporated with the plasmids expressing the eRF3a variants. Three days later, the effect on stop codon readthrough was analyzed by measuring the β-galactosidase activity. Cells electroporated with the empty vector pSuper and re-electroporated with plasmid pBK-CMV served as a negative control (Fig. 3A, lane sup/pBK). Cells electroporated with plasmid expressing si-3a1 and re-electroporated with plasmid pBK-CMV served as standard for readthrough level promoted by eRF3a depletion (Fig. 3A, lane 3a1/pBK). For each electroporation experiment, the amount of endogenous eRF1 and eRF3a overexpressed proteins was verified by Western blot analysis. As shown
in Fig. 3B, all of the eRF3a variants were expressed at the same level. Our results show no significant differences in the readthrough efficiencies whatever the eRF3a variant overexpressed (Fig. 3B). This suggests that the length of the glycine stretch encoded by the GGC repeat has no detectable effect on eRF3a activity in translation termination.

**Discussion**

eRF3 is a critical protein, playing a role in several points of cell regulation. The N-terminal domain of eRF3a has been shown to participate in essential protein interactions necessary for different eRF3a functions, namely the formation of the translation termination complex (10), coupling termination with re-initiation in successive cycles of translation (23) and as an initiator of NMD (24). Apart from its canonical roles in translation, the N-terminal domain of eRF3a was also reported to interact with various proteins with different biological functions (9) and also to act as a regulator of apoptosis, being proteolytically processed into an isoform that contains a conserved N-terminal IAP-binding motif (25). As it is implicated in essential cellular processes, modifications in the amino-terminal domain of eRF3a might be of crucial relevance.

We have previously showed that the longer allele (12-GGC) of the GGCn polymorphism in eRF3a/GSPT1 exon 1 is present in 8% of gastric cancer patients (11/139), and thus is associated with ~20-fold increased risk of gastric cancer (15). We also demonstrated that eRF3a/GSPT1 mRNA expression is up-regulated in 70% of the intestinal type gastric tumors analysed (6). However, the correlation between the patient genotype and the levels of eRF3a/GSPT1 expression was not established.

Herein, we report the presence of the 12-GGC allele in 5% (7/137) of the breast cancer patients, in contrast with zero in the control population (0/135). Given the rarity of
the allele, the statistical results need to be strengthened by replication in other populations. When both gastric (15) and breast cancer populations are grouped together and compared with the control population, the 12-GGC allele is present in 6.52% of the cancer cases, with an allele frequency of 3.8%, corresponding to a 15-fold increased risk for cancer (OR=15.49; 95% CI=0.93-257.43). Therefore, we propose that the presence of the 12-GGC allele, by itself, may provide a new useful marker for cancer susceptibility.

Relative quantification of mRNA in breast tumor tissues revealed an overexpression of eRF3a/GSPT1 in 44% of the tumors analysed. It is known that cell growth and proliferation rates depend critically on the rate of protein synthesis. To investigate if variations in eRF3a/GSPT1 gene expression pattern are quantity adjustments dependent on the cell translation rates, we quantified the levels of expression of the other translation termination factors. Because eRF1 is the major factor in translation termination, we analysed the pattern of eRF1 gene expression assuming that it is dependent on cell translation rates. No variations between normal and tumor tissues were detected in 44% of the patients analysed. Moreover, there are no correlations between eRF1 and eRF3a/GSPT1 transcript levels. In what concerns eRF3b, an isoform of eRF3a, no transcripts were detected in the majority of the patient tissues analysed and no correlation between eRF3a/GSPT1 and eRF3b/GSPT2 expression was detected. Therefore, there is no parallel between eRF3a/GSPT1 transcript levels and additional cell requirements for increased translation.

Our results provide the first indication suggesting that the length of the GGC repeat is associated with the variations of eRF3a/GSPT1 transcript levels in tumor samples. The longer allele (12-GGC) was only found in patients with eRF3a/GSPT1 overexpression in tumor samples. In contrast, the shorter allele (7-GGC) was mostly associated to patients with eRF3a/GSPT1 underexpression.

A number of hypotheses can be raised to explain the association of the 12-GGC allele with cancer development. It can involve a dominant gain of function effect by shifting the protein’s conformation, thus altering its function or achieving toxicity. Loss of function can also occur, by interfering with DNA methylation, RNA processing or translation efficiency. As a first approach, we looked for differences in the efficiency of eRF3a with different lengths of polyglycine stretches in the N-terminal domain. However, the GGC repeat length showed no effect in eRF3a activity, in what concerns its role as a translation termination factor.

It is known that protein synthesis is coupled with cell cycle progression and is regulated in response to nutrient availability and mitogenic stimulation. However, the transcription pathways activated do not stimulate the translation of all the mRNAs equally (3,26). The expression of the components of the translation machinery might be selectively regulated, increasing the translation rate of specific oncogenic transcripts (26,27). Our recent results revealed that eRF3a depletion inhibited translation and cell cycle progression in HCT116 cell line via mTOR pathway inhibition (28). After activation by mTOR, S6K phosphorylates the ribosomal protein S6, leading to an increase in translation of a subset of mRNAs (29). Therefore, it is tempting to speculate that eRF3a overexpression detected in different kinds of tumors might be responsible for mTOR activation, leading to differential expression of specific targets and consequent malignant transformation. It is noteworthy that the 12-GGC allele was only detected in patients who are simultaneously RE and RP positive, which are important markers usually taken in consideration for prognostic evaluation and therapeutic decisions (22).

Understanding eRF3a/GSPT1 gene regulation and its relation with cell cycle progression and cellular proliferation may have prognostic value and potential therapeutic applications. A better knowledge of the influence of the polyglycine stretch on the role of eRF3a will allow to establish whether eRF3a could be used as a new biomarker for breast and other types of hereditary cancer.

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References


