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Original Communication

Human epidermal growth factor receptor in malignant development of leukocytes: expression of a truncated transcript in Burkitt B lymphomas

G. Padron[1], C. S. Moraes[1], I. J. F. Motta[2] and C. S. Freitas[1,*]

[1]National Cancer Institute of Rio de Janeiro, CPQ Research, Rua Andre Cavalcante 37,
[2]National Cancer Institute of Rio de Janeiro, HC1 Blood Bank, Praça Cruz Vermelha 23,
CEP 20231-050, Center, Rio de Janeiro, RJ, Brazil

ABSTRACT

We searched for Human Epidermal growth factor Receptor (HER)-related transcripts using 14 human leukemia/lymphoma cell lines, assuming that cell lines grown *in vitro* are representative of malignant leukocyte lineages and stages of development. A unique truncated HER1 transcript was observed in 3 out of 4 Burkitt B lymphomas and 1 Erythroblastoid leukemia (K562), which was absent in healthy peripheral blood mononuclear cells and in pre-B leukemia cell lines. In agreement with the reported down-regulation of HER1 in normal leukocyte development, this finding suggests a resumption of HER1 expression during B cell malignant lymphoma development as a secondary and relatively late event, posterior to IgH rearrangement. Healthy mononuclear cells down-regulated HER1, but were positive for its ligands and for HER2, suggesting underestimation of the presence of "HER system" in mature peripheral blood cells.

KEYWORDS: B lymphocyte development, EGFR, Human EGF Receptors, HER-focused therapies, leukopoiesis

ABBREVIATIONS

c-erbB, cellular-erythroblastosis B; EGF, Epidermal Growth Factor; EGFR, Epidermal Growth Factor Receptor; G3PDH, Glucose 3-Phosphate Dehydrogenase; GALT, Gut-Associated Lymphoid Tissue; HB-EGF, Heparin-Binding EGF; HER, Human Epidermal growth factor Receptor; NRG4, Neuregulin 4; TGF-alpha, Transforming Growth Factor Alpha; vErbB, viral oncogene involved in chicken Erythroblastoid leukemia

*Corresponding author: csondermannf@inca.gov.br

INTRODUCTION

The "EGF/EGFR system" (Epidermal Growth Factor/Epidermal Growth Factor Receptor) plays an essential role in development and is maintained active during adult life in most tissues. Mature blood cells, however, have been reported as an exception [1, 2]. This suggests that during normal leukopoiesis, cells might down-regulate the EGFR gene expression from a given stage onwards, as described for erythroid progenitor development [3].

Cellular ErbB (cErbB; EGFR; HER1) was first described as the counterpart of a viral oncogene involved in chicken Erythroblastoid leukemia (vErbB) [4]. However, knowledge concerning HER-family-related participation in malignant blood cell development remains limited and controversial, despite some important reports. For example, the overexpression of HER2 was observed in multiple myeloma cells [5], even before Mahtouk *et al*. [6] demonstrated the influence of the "HER system" on myeloma cell growth. Regarding B cell malignant growth,

Bühring *et al.* [7] described HER2 expression in acute lymphoblastic leukemia and in chronic myeloid leukemia, the latter during B blast crisis. Ebi *et al*. [8] reported the growth of lymphomas of gut-associated lymphoid tissue (GALT) stimulated by the HER4 ligand Neuregulin 4 (NRG4), although this growth factor was absent in the leukemia cell lines they tested [9].

Besides transcript regulation, mutations in HER1 and HER2 genes have also been reported in human cancers [10, 11]. However, concerning blood malignancies, HER1 mutations were not verified in a study involving 88 acute leukemia patients [12].

In this work, we used human leukemia/lymphoma cell lines, representative of differentiation lineages and stages of development [13], for screening transcript expression, and mononuclear blood cells from healthy individuals as negative control. Expression of the HER1 transcript was observed in 3 out of 4 Burkitt B lymphoma cell lines, but was absent in negative controls and pre-B leukemia cell lines. Also a unique truncated form of this transcript was detected. Furthermore, both HER1 ligands and HER2 transcripts were observed in malignant cells, making an autologous signaling loop possible. The transcripts of these ligands were also expressed in HER1-negative normal blood cells, suggesting a previously unsuspected physiological role for these "orphan" molecules in healthy leukocytes.

MATERIALS AND METHODS

Obtaining blood mononuclear cells from healthy individuals

Approximately 10 mL samples of citrate-EDTA (ethylenediaminetetraacetic acid)-anticoagulated peripheral blood from healthy individuals were obtained from the National Cancer Institute Blood Bank, in accordance with Research Ethics Committee protocols #130/08 and 51/11. Mononuclear cells were collected after centrifuging blood for 45 min at 450 *g* through Ficoll-Hypaque (d = 1.077) (Amersham Biosciences, SW), followed by two washings with PBS (Phosphate-Buffered-Saline)-EDTA.

Cell culture

The cell lines used were: 3 myeloid leukemias (granulocytic HL60, erythrocytic K562, monocytic U937), 4 Burkitt lymphomas (Daudi, Ramos, Raji and Namalwa), 1 pre-T cell leukemia (CCRF-CEM) and 6 pre-B leukemias (ALL-PO, CEMO1, REH, RS4; 11, NALM6 and 697). The cells were grown in RPMI 1640 medium (SIGMA, USA) containing 10% v/v fetal bovine serum (GIBCO, USA), penicillin and streptomycin and maintained exponential growth up to collection. After collection, the cells were washed twice with Hank's Salt Solution before RNA was extracted using TRIZOL (Invitrogen, USA). The A431 cell line (epidermoid carcinoma of the vulva) was used as positive control for HER receptors and ligands and was grown in Dulbecco/Ham F12 (SIGMA) with 1 ng/ml murine EGF (SIGMA).

RT-PCR (Reverse transcriptase polymerase chain reaction)

cDNA was obtained using 5 µg total RNA with Oligo dT (0.5 µg/µL) and SuperScript^{TM}II in the presence of RNAse inhibitor (RNAseOUT) (Invitrogen). Amplifications were performed using Taq DNA polymerase (Promega, USA); the primer pairs and annealing temperatures are presented in Table 1. The PCR cycles were as follows: an initial denaturation at 95 °C for 3 min was followed by 35 cycles of denaturation (95 °C for 45 sec), annealing at specific temperatures (for 35 sec) and extension (72 °C for 1 min). A final extension of 10 min at 72 °C was performed, and then the reaction was maintained at 4 ^{o}C. The products were analyzed on 2% agarose gels in TAE buffer in the presence of Ethidium bromide.

Protein immunoprecipitation and western blot

The surface proteins of approximately 10^7 NALM6 cells were biotinylated as follows [14]. After washing in BIWA's buffer (PBS with 1 mM MgCl 2, 0.1 mM CaCl 2, pH 7.2) and ressuspension (10^7 cell/mL) in 10 mM Borate buffer (pH 8.8) with 150 mM NaCl 50 µg/mL plus freshly added Sulpho-Biotin (Pierce, USA), the cells were agitated for 15 min at room temperature. The reaction was blocked with 25 mM L-Lysine in BIWA's buffer and the cells were washed three times with L-Lysine solution before preparing the cell protein extract. The cells were lysed in RIPA buffer (Tris 50 mM, NaCl 0.15 M, EDTA 1 mM, pH 7.4) containing 1% Triton X100, 0.5% w/v Sodium deoxycholate, 0.1% w/v SDS (Sodium Dodecyl Sulfate) and the

Table 1. Primer pairs, annealing temperatures, and expected band sizes.

	Primer pairs	Annealing temperatures	Expected band sizes
G3PDH	F- 5'- TGA CCC CTT CAT TGA CCT CA 3' R- 5'- AGT CCT TCC ACG ATA CCA AA 3'	55 °C	419 bp
HER1 5'UTR to exon 7	F- 5'- CCA GTA TTG ATC GGG AGA GC 3' R- 5'- GGA CAC TTC TTC ACG CAG GT 3'	55 °C	932 bp
HER1 exon 2 to 3	F- 5' - TAA CAA GCT CAC GCA GTT GG 3' R- 5'- GTT GAG GGC AAT GAG GAC AT 3'	55 °C	176 bp
HER1 exon 1 to 8	F- 5' - GGG CTC TGG AGG AAA AGA AA 3' R- 5' - AGG CCC TTC GCA CTT CTT AC 3 '	55 °C	929 bp vIII 128 bp
HER1 exons 7 to 11	F- 5' - ACC TGC GTG AAG AAG TGT CC 3' R- 5'- TTG TCA AAG GCA TGG AGG TC 3'	55 °C	398 bp
HER1 exon 11 to 14	F- 5'- GAC CTC CAT GCC TTT GAG AA 3' R- 5'- TTC TCC ACA AAC TCC CTT GG 3'	55 °C	410 bp
HER1 exon 24 to 28	F- 5'-CAG CGC TAC CTT GTC ATT CAG 3' R- 5'- TCA TAC TAT CCT CCG TGG TCA 3'	55 °C	726 bp
HER2 exon 15 to 18	F- 5' -TAC ATG CCC ATC TGG AAG TT 3' R- 5' - AGC TCC GTC TCT TTC AGG AT 3'	55 °C	332 bp
EGF exon 19 to 21	F- 5' CCT TGG GGA AGA TGG CCA CCA 3' R- 5' CAT GCA GAC AGC CAC CAC CAT 3'	55 °C	240 bp
TGF-alpha 5'UTR to exon 4	F- 5'- CTG CCC GCC CGC CCG TAA AA 3' R-5'- CCG CAT GCT CAC AGC GTG CA 3'	65 °C	274 bp
HB-EGF exon 1 to 3	F- 5' - GGT GCT GAA GCT CTT TCT GG 3' R- 5' - ACA TGG GTC CCT CTT CTT CC 3'	55 °C	304 bp

EGF, Epidermal Growth Factor; **G3PDH**, Glucose 3-Phosphate Dehydrogenase; **HB-EGF**, Heparin-Binding EGF; **HER**, Human Epidermal growth factor Receptor; **TGF-alpha**, Transforming Growth Factor alpha.

protease inhibitors Aprotinin, Pepstatin, Leupeptin and PMSF (Phenyl Methyl Sulfonyl Fluoride), for 30 min at 4 ^{o}C, followed by centrifugation at 14,000 *g* for 20 min at 4 ^{o}C. The supernatants were incubated overnight with 1 µg of rabbit anti-HER2 monoclonal antibodies (Abs) (clone 29D8, Cell Signaling), then with beads of Protein A-conjugated Sepharose-CL4B (SIGMA) for 3 h, with stirring. After three washings with lysis buffer, the beads were boiled with SDS-PAGE Sample Buffer [15] and the supernatants were analyzed on a 7.5% separating acrylamide gel using Kaleidoscope Prestained MW Standards (BIO-RAD, USA). The proteins were transferred to a Hybond-C Extra Nitrocellulose membrane (Amersham, USA) in Towbin buffer with 20% methanol. The membrane was blocked in 5% w/v skimmed milk in Tris-NaCl buffer with 0.1% Tween 20 for 1 h, before Alkaline Phosphatase-conjugated Extravidin (SIGMA) was added, and incubated for 1 h. The biotinylated proteins were revealed by the addition of the substrate BCIP-NBT (5-Bromo-4-Chloro-3-Indolyl-Phosphate with Nitro Blue Tetrazolium) (SIGMAFAST).

RESULTS

Figure 1A shows the broad expression of transcripts for HER2 in most of the leukemia/lymphoma cell

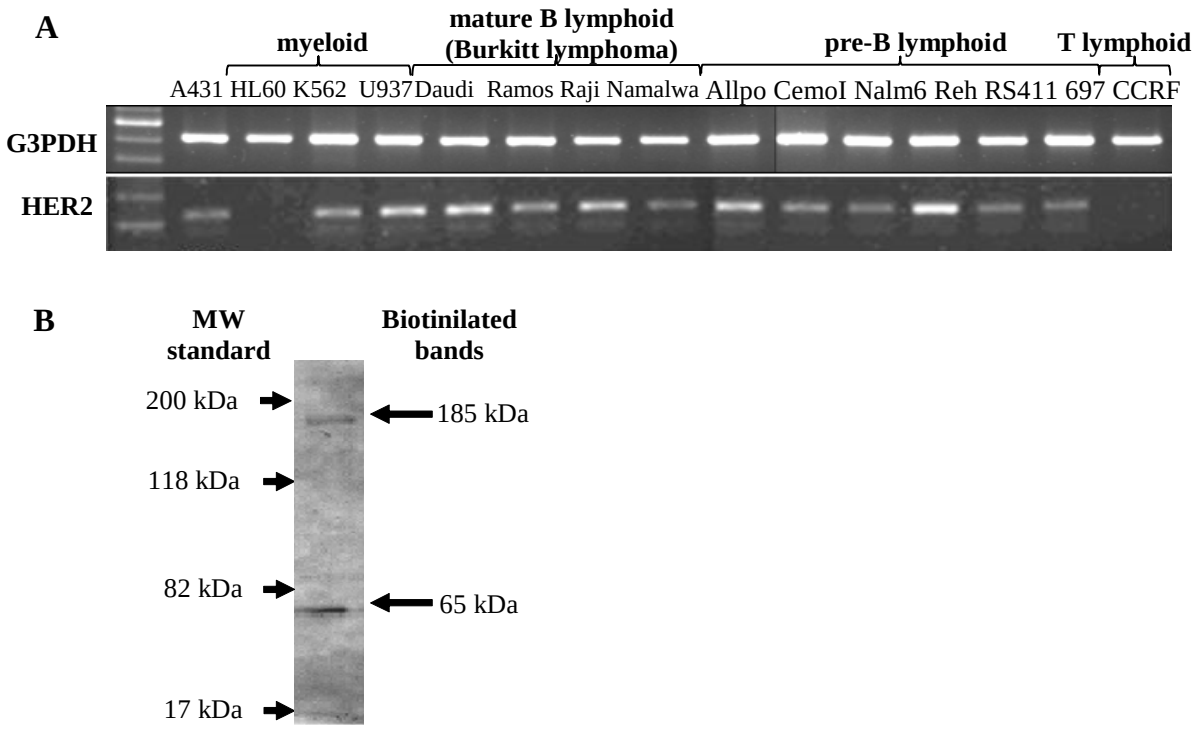

Figure 1. A, RT-PCR to detect HER2 transcripts in leukemia/lymphoma cell lines. A431 carcinoma represents positive control for HER2 expression. Glucose 3-Phosphate Dehydrogenase (G3PDH) represents control for housekeeping genes. **B, Western blot for HER2 protein detection at the surface of Nalm6 Pre-B leukemia cell line.** The surface of living cells was biotinylated and the protein extract submitted to immunoprecipitation with anti-HER2 monoclonal antibodies plus bead-conjugated protein A. The bands were revealed by alkaline phosphatase-conjugated Extravidin plus BCIP-NBT. Two bands were observed, one of approximately 185 kDa, as expected for HER2, and a smaller one of approximately 65 kDa, of undetermined identity.

lines evaluated (12 out of 14), the exceptions being the granulocytic myeloid HL60 and the T lymphoid CCRF-CEM lines. The identity of the HER2 amplicons was confirmed by DNA sequencing (data not shown) and corroborated by ascertaining its translation into protein, using immunoprecipitation and Western blot. Anti-HER2 immunoprecipitates from a protein extract of surface-biotinylated NALM6, a prototypic pre-B leukemia [13], showed a band of the size expected for HER2 (approximately 185 kDa), as well as an additional band of 65 kDa that remains to be identified (Figure 1B). Thus, as shown with this cell line, the receptor was available at the cell surface for hetero-dimerization and protein tyrosine kinase action.

In the case of HER1 expression, however, a more selective pattern was observed. The HER1 transcript region between exons 11-14, that was tested initially, was present in Burkitt B lymphomas (3 out of 4, except Namalwa), and in the K562 erythroblastoid cell line, but was absent in the other cell lines, including the 6 pre-B leukemias (Figure 2A).

When different primer pairs were used in the analysis of HER1-positive cells lines, truncated transcripts were detected (Figure 2B). In fact, the band comprising 5'UTR to exon 7 was shortened. The codifying portion for the first, most distal, ligand-binding region of the extracellular moiety of the protein molecule was lacking in all four of the positive cell lines, while the codifying portion for the second ligand-binding region, comprising exons 7-11, was present in all of them. The codifying portion of the intracellular moiety comprised between exons 24-28 was transcribed in Daudi and Ramos lymphomas, but were not detected in Raji lymphoma and K562 erythromyeloid cells.

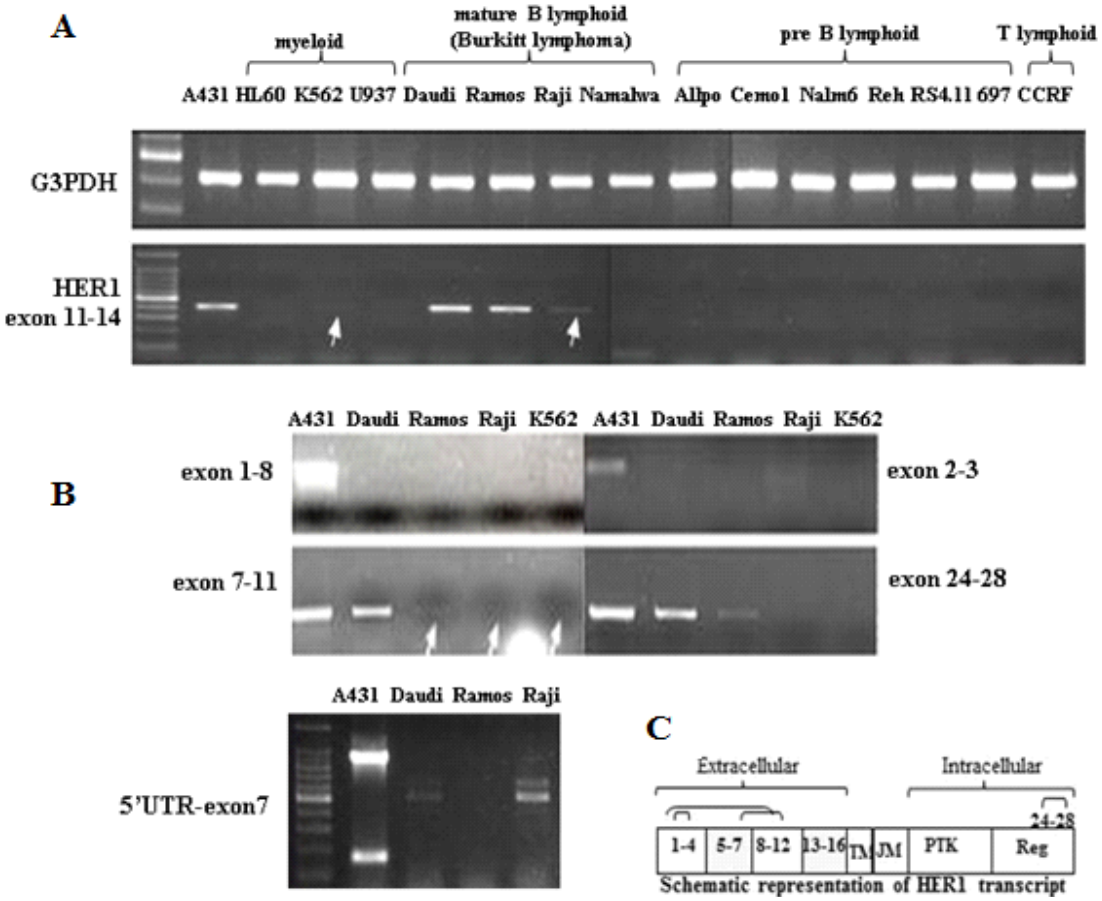

Figure 2. RT-PCR to detect transcripts for HER1 and its truncated regions in leukemia/lymphoma cell lines. A, initial screening, assessing exons 11-14; **B,** analysis of the specific regions between exons 1-8, 2-3, 7-11, 24-28, and 5'UTR-exon 7. **C,** schematic representation of HER1 transcript marking the exons studied, as shown in **B**. A431 carcinoma represents positive control for HER1 expression. Glucose 3-Phosphate Dehydrogenase (G3PDH) represents control for housekeeping genes. White arrows indicate faint positive bands.

Concerning HER1 ligands, transcripts for EGF, HB-EGF (Heparin-Binding EGF), and TGF alpha (Transforming Growth Factor alpha) were respectively present in 11 out of 14, 6 out of 14, and 12 out of 14 cell lines of varying phenotypes (Figure 3). This suggests a possible ligand-mediated activation of HER1 in positive cell lines, since even the truncated molecule still retains the second extracellular ligand-binding region.

Mature mononuclear blood cells from healthy individuals, collected to provide negative controls, were negative for HER1 transcripts as expected, but showed the presence of transcripts for HER1 ligands and HER2 (Figure 4; shown 5 out of 8 positive individuals), which did not seem to be developmentally down-regulated.

DISCUSSION

We searched for HER-related transcripts using 14 human leukemia/lymphoma cell lines, assuming that cell lines grown *in vitro* are representative of malignant leukocyte lineages and stages of development [8, 13, 16-18].

HER1 transcript expression was observed in 3 out of 4 Burkitt B lymphomas and 1 erythoblastoid leukemia (K562), but was absent in healthy peripheral blood mononuclear cells and in pre-B leukemia cell lines. In agreement with the reported down-regulation of HER1 in normal leukocyte development [1, 2], this finding suggests a resumption of HER1 expression during B cell malignant development as a secondary and

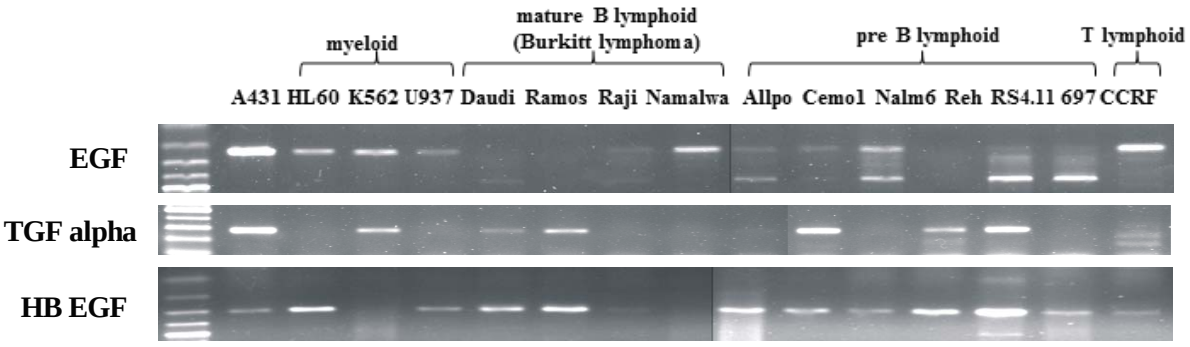

Figure 3. RT-PCR to detect transcripts for HER1 ligands in leukemia/lymphoma cell lines. A431 carcinoma represents positive control for HER1 ligands expression. Transcripts for EGF (Epidermal Growth Factor), TGF alpha (Transforming Growth Factor alpha), and HB-EGF (Heparin-Binding EGF) were broadly present in malignant blood cell lines.

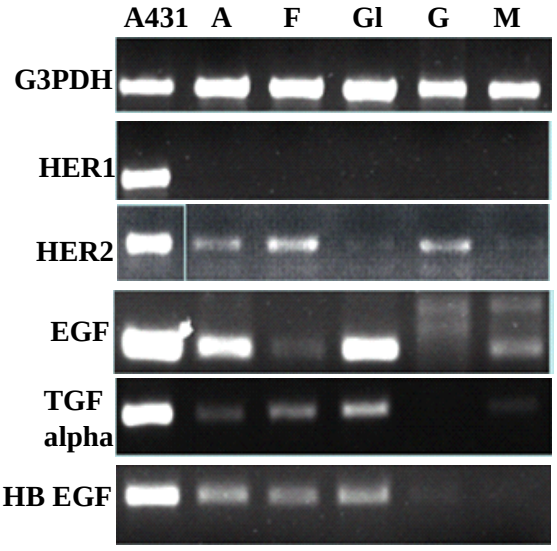

Figure 4. RT-PCR to detect transcripts for HER receptors and ligands in mononuclear cells from the peripheral blood of healthy donors. A431 carcinoma represents positive control. Glucose 3-Phosphate Dehydrogenase (G3PDH) represents control for housekeeping genes. White arrows indicate faint positive bands. Transcripts for HER2 were present in 5 out of 5 individuals, HER1 was absent, and HER1 ligands EGF (Epidermal Growth Factor), TGF alpha (Transforming Growth Factor alpha), and HB-EGF (Heparin-Binding EGF) were broadly expressed.

relatively late event. This developmental hypothesis was further supported by our unpublished data using blood cells from patients with B-LLA, which were all HER1-negative.

Burkitt B lymphomas show the characteristic phenotype of nonactivated mature B lymphocytes, their transcriptomes revealing the signature of germinative center lymphocytes [17, 19]. The primary malignant event in this pathology, either in the African (endemic) or American (sporadic) types, occurs during the IgH rearrangement, by translocation between IgH and c-myc genes [18, 19], after which the HER1 truncated transcript is presumably expressed. It seems improbable that the absence of HER1 transcript in Namalwa cells can be explained on the basis of its developmental characteristics. Daudi, Raji and Namalwa originated from African lymphomas and are associated with EBV infection, while Ramos belongs to the group of American lymphomas, EBV-free and usually developmentally more mature than the endemic forms [20].

The truncated HER1 transcripts described herein did not correspond to the variants previously reported in human epithelial tumors, for example, the HER1 vIII variant, truncated between exons 1-8, which produces a 128 bp transcript band [21]. Active truncated receptors have been described among members of the HER family, in some solid tumors and malignant cell lines [10, 22-23]. Forms truncated for the extracellular region (lacking N-terminal protein regions) can still associate with full length forms of HER receptors, inducing phosphorylation and activation of mitogenic signaling [24]. Even the deletion of trans-phosphorylation sites does not hamper signal

transduction; for example, if the whole intra-cytoplasmatic HER1 tail is lost, EGF binding to its extracellular moiety can still induce dimerization with a HER2 chain and potent mitogenic signals [25].

Here, transcripts for HER1 ligands were observed in the HER1-positive malignant cells, together with transcripts for HER2, permitting a putative autologous-stimulated growth. HER2 expression did not seem to be developmentally down-regulated, since it was also present in mature blood cells from healthy individuals.

Curiously, healthy mononuclear cells were negative for HER1 receptors, but positive for its ligands. In these cells, the "orphan" ligands putatively display alternative roles related to a paracrine interaction in homeostasis or to immune responses. Another hypothesis that could explain why the "orphan" ligands are maintained in healthy mononuclear cells is related to the usual translation of transmembrane pre-pro-peptides from growth factor transcripts (as reported in other cells), which, following metalloproteinase cleavages, generate active intracellular fragments able to perform numerous functions, including acting as transcription factors [26]. The findings reported here suggest that the presence of "HER system" in healthy blood cell physiology has been underestimated and deserves further investigation.

Ebi *et al*. [8] were unable to detect transcripts for HER1 ligands in lymphoma cell lines, but verified NRG4- and HER4-related autologous-induced growth when studying patients' GALT and follicular lymphomas. They also observed tyrosine phosphorylation of HER4 induced by recombinant NRG4 in the Burkitt lymphoma cell lines Raji and Daudi. Thus, together with the reports on malignant myelomas [6], malignancies of mature B lymphocyte phenotype would figure as targets for HER-focused therapies initially developed for epithelial cancers.

CONCLUSIONS

The results obtained suggest a resumption of HER1 transcript expression in late malignant development of B lymphocytes and include Burkitt lymphoma among the malignancies of mature B lymphocyte phenotype that represent putative targets for HER-focused therapies. In addition, we report a unique truncated version of the HER1 transcript receptor. Furthermore, maintenance of the expression of HER1 ligands and HER2 in normal development of leukocytes suggests that the presence of "HER system" in healthy blood cell physiology is underestimated.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no conflicts of interest.

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