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Cloning and expression of a novel type (III) of human γ-glutamyltransferase truncated mRNA

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Abstract We report the characterization of a novel human γ-glutamyltransferase mRNA type. This type III mRNA differs from type I and type II mRNAs previously described by several point mutations and the presence of an unspliced 81 bp intron in the open reading frame. Further, type III mRNAs are truncated ones and are tissue and pathology specifically expressed. In fact, type III mRNAs are present in human placenta, sigmoid, lung and in 50% of acute lymphoblastic leukemia blood cells but they are never found in healthy lymphocytes.

Key words: γ-Glutamyltranspeptidase; Multigenic family; Truncated mRNA; Leukemia

1. Introduction

γ-Glutamyltranspeptidase (EC 2.3.2.2, GGT) is a plasma membrane-bound heterodimeric enzyme that catalyses the hydrolysis or transfer of the γ-glutamyl moiety from glutathione and other γ-glutamyl-containing compounds to an amino acid or dipeptide, to water or to glutathione [1,2].

Human GGT is a multigenic family composed of at least 7 loci [3]. Five of them are located on chromosome 22 [4] in the *bcr* region (break point cluster region) which is involved in chromosomal breaks and rearrangements associated with various diseases [5].

Human GGT mRNAs expressed in placenta [6], pancreas [7], fetal liver [8], lung [9] and HepG2 hepatoma [10] have been described previously and show a unique ORF. This ORF encodes for a single polypeptide precursor which upon cleavage during the post-translational process gives rise to the two non-identical subunits of the mature enzymes (large subunit 55–60 kDa, small subunit 21–30 kDa) [11]. These mRNAs, named type I mRNA, seem to be encoded by our ubiquitously expressed GGT gene (gene 6) [3].

Wetmore et al. [9] have characterized another type I mRNA expressed in human lung which is truncated in the sequences encoding the large subunit. The sequences encoding the small subunit are identical to type I mRNA.

Another mRNA, named type II, is encoded by gene 3 and expressed in human kidney and has been described by Pawlak et al. [12]. It contains several point mutations in comparison to type I mRNA. Actually, no protein translated from this mRNA has been identified.

It has been reported that at least three more GGT genes are expressed in human tissues and cell lines although no further characterization of the corresponding transcipts has been attempted [3]. Among them, gene 1 transcripts seem to be the most interesting as they were found in placenta, which presents a high GGT activity, in lung and sigmoid. Furthermore, GGT activity is known to be modulated in several disorders including cancer diseases [13] and also during blood cell differentiation [14]. In acute lymphoblastic leukemia, GGT activities in white blood cells are modified [15].

The aim of our studies was first to clone and to characterize the GGT type III mRNA. Furthermore, we studied the expression of type I and type III mRNAs in healthy and ALL subjects.

2. Materials and methods

2.1. Biological samples

Peripheral blood was collected in EDTA-Vacutainer tubes (Beckton-Dickinson, USA) according to a protocol approved by the 'Comité de Protection des Personnes'.

Normal blood was obtained from 10 healthy volunteers between 20 and 30 years old.

Lymphocytic fractions were obtained by Ficoll-Paque (Sigma, USA) gradient centrifugation [16] from blood of 17 admitted patients both male and female (2–15 years old) (department of Prof. D. Sommelet at the Médecine Infantile II, Hôpital d'Enfants de Brabois, Vandoeuvre-lès-Nancy, France) who had a hematologic diagnosis of acute lymphocytic leukemia (5 bone narrow relapses, 1 remission and 11 diagnoses). Total RNA was extracted using the guanidinium thiocyanate method [17].

Hematopoietic cell lines HL60, U937 and K562 were subcultured in RPMI-1640 medium (Gibco-BRL, France) supplemented with 5% fetal calf serum (Boehringer-Mannheim, France) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Total RNA was extracted from confluent cells as described previously.

2.2. Reverse transcription

The conversion of RNA to cDNA was carried out in final volume of 50 μl containing 1–5 μg of total RNA extracted from the lymphocyte fraction, cultured cells or placenta, 50 mM Tris-HCl, pH 7.6, 70 mM KCl, 10 mM $MgCl_2$, 4 mM dithiothreitol, 10 ng oligo-d$(T)_{12-18}$ primer (Gibco-BRL, France), 1 mM each dNTP, 40 U of 'RNA guard' (Pharmacia, France) and 200 U of MMLV reverse transcriptase (Gibco-BRL, France). Before adding RNA guard and reverse transcriptase, the mixture was heated at 70°C for 5 min and the reaction was quenched on ice. Reverse transcription was then carried out at 37°C for 90 min and stopped by cooling on ice.

2.3. DNA amplification and cloning

cDNA was amplified with Taq DNA polymerase (Boehringer-Mannheim, Germany) in a thermal cycler (Perkin Elmer-Cetus,

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Abbreviations: ALL, acute lymphoblastic leukemia; GGT, γ-glutamyltransferase; kb, kilobases; ORF, open reading frame; RT, reverse transcriptase; 1×SSC, 0.15 M NaCl/0.015 M sodium citrate.

The sequence reported here was submitted to EMBL under the accession number X98922.

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USA). Samples were prepared with 5 μl of the cDNA solution in a total volume of 100 μl containing the following PCR mixture: 1×PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine), 250 μM each dNTP and 1 μM of each 5′ and 3′ primer. The mixture was overlaid with mineral oil (Sigma, France) and after initial denaturation at 96°C for 6 min, the Taq DNA polymerase was added, and then incubated for amplification. Steps were: denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and extension for 2 min at 72°C. GGT type I mRNA specific PCR reactions were conducted with primers Amp 1 (5′-TTACCTACTGAAGTCGAGAGG-3′) and Amp 2 (5′-TCGGCTTGGTCTCCAACATCT-3′) for 35 cycles. GGT type III specific reactions were performed with Amp 1 and Amp 5 (5′-AAAGGGGTGACACATATCAG-3′) for 35 cycles.

Aliquots of final products were separated on a 3% agarose gel (NuSieve, FMC, USA), electrophoresed, visualized under UV illumination and blotted onto nylon membrane (Hybond N, Amersham, USA).

Rapid amplification of cDNA 3′ ends (3′ RACE) was performed as in the reverse transcription using 5 μg of human total placental RNA and R0-dT (5′-CCGAATTCAA $d(T)_{17}$-3′) as primer. PCR amplifications with Amp 1 as 5′ primer and R0-dT as 3′ primer (denaturation for 1 min at 94°C, annealing for 1 min at 55°C and extension for 2 min at 72°C) were performed under the conditions described above. Final products were separated on 1% agarose, UV visualized, purified and cloned into *Sma*I digested pSK vector according to standard procedures.

For 5′ RACE experiments, 2 μl of human placental cDNA (Clonetech, USA) were first amplified with Anchor (5′-GGTTCGGCCCACCTCTGAAGGTTCCAGAATGCAT-3′) (Clonetech, USA) as 5′ primer and Amp 5 as 3′ primer for 40 cycles (denaturation for 45 s at 94°C, annealing for 45 s at 55°C and extension for 2 min at 72°C). 5% of these amplification products were reamplified using the same conditions with Anchor as 5′ primer and Amp 5 or Amp 8 (5′-GCAGTCTCTAGACCCGGG-3′) as specific 3′ primer.

The products were separated on a 1.5% agarose gel, purified and cloned into the pCRII vector (TA cloning kit, Invitrogen, USA).

Nucleotide sequence determination was performed using Sanger's method [18] with the T7 Sequencing kit (Pharmacia) and (α-^{32}P)-labeled dATP (Dupont-Nemours, USA).

2.4. Oligonucleotide hybridizations

The positive control for gene 1 hybridization was the gene 1 *Bgl*II restriction fragment cloned in pSK and described by Courtay et al. [3].

Blots were prehybridized for 2 h at 65°C in 3×SSC, 10×Denhardt's, 50 μg/ml salmon sperm DNA and 0.1% SDS; hybridization was overnight at 55°C in the same solution with a type I (mut 6: 5′-CCAACGAGTTTGGGGTACCC-3′) or type III (mut 1: 5′-CTCTCGTCAATGTGCCCGAC-3′) mRNA specific (γ-^{32}P)-labeled oligonucleotide. After post-hybridization washing in 3×SSC, 0.1% SDS at 55°C, membranes were used to expose a film at −80°C for different times.

3. Results

3.1. Type III GGT mRNAs are specifically amplified with Amp 1 and Amp 5 primers

Courtay et al. [3] have partially described the genomic sequence encoding GGT type III mRNA, and they have shown a point mutation at the 5′ end (the normal GT 5′ donor site is replaced by AT) of an 81 bp intronic sequence that should result in an unspliced intron. The oligonucleotide Amp 5 hybridizes at the 5′ end of this putative unspliced intron and therefore should be specific for type III GGT mRNAs. Thus, Amp 1 and Amp 5 were used as specific primers for RT-PCR reactions on placenta and sigmoid RNA where the expression of this GGT gene has been previously shown [3]. As shown in Fig. 1A, the amplification products with Amp 1 and Amp 5 primers were about 210 bp which is in agreement with the theoretical length. Furthermore, these products specifically hybridized with the gene 1 (type III) specific oligonucleotide (Fig. 1B, lane 2) while no hybridization occurred when the gene 6 (type I) specific oligonucleotide was used instead. Thus, the RT-PCR reactions with Amp 5 and Amp 1 primers are specific for type III GGT mRNA.

3.2. Partial cloning of GGT type III mRNA

3′ RACE and 5′ RACE were used to clone type III GGT mRNA.

3′ RACE was performed using Amp 1 as the 5′ primer. PCR products were cloned in the *Sma*I digested pSK phagemid, restriction mapped and sequenced. One clone was found to contain a 620 bp insert whose sequence was completely identical to the genomic DNA [3] and corresponded to the exons encoding the small GGT subunit (Fig. 2). As expected,

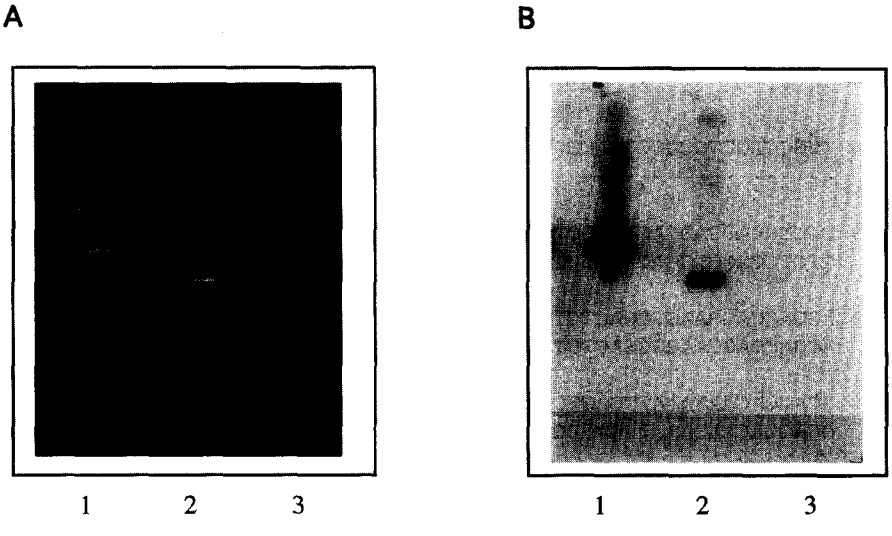

Fig. 1. Amp 1-Amp 5 amplification of placental cDNA and gene 1 specific hybridization. 5 μg total placental RNA were subjected to RT-PCR amplification with Amp 1 and Amp 5 as primers and hybridized with gene 1 specific oligoprobe. Lanes: 1, a positive control for gene 1 hybridization; 2, RT-PCR product obtained with placental RNA as template; 3, ΦX DNA digested by *Hae*III. (A) PCR products were separated on 1.5% agarose and visualized by UV light. (B) The gel used in (A) was blotted. Hybridization with a gene 1 specific oligonucleotide was performed at 55°C; washing of the membrane was carried out at 60°C.

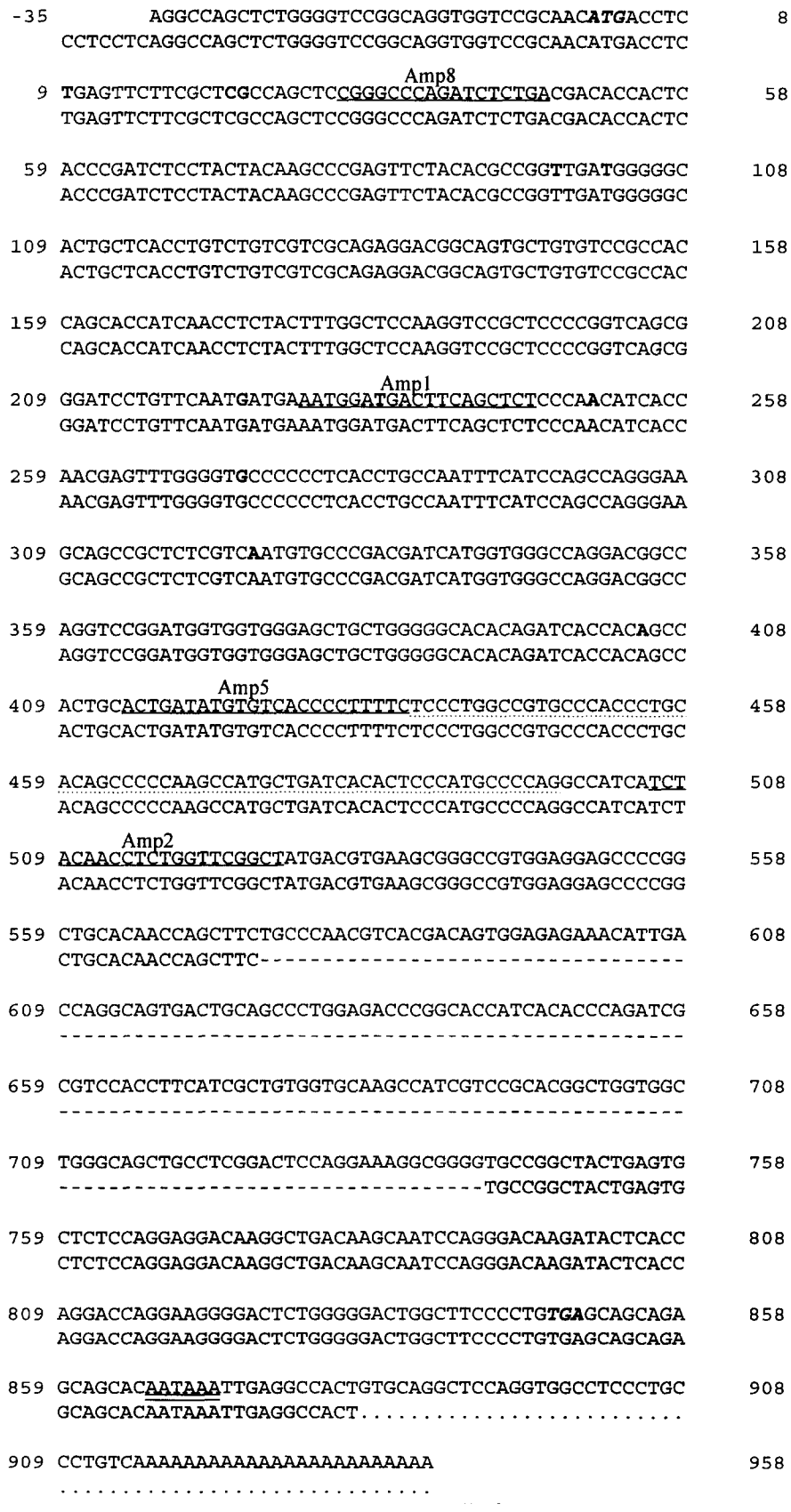

Fig. 2. Comparision of gene 1 genomic and RACE products sequence. The upper line contains the type III cDNA sequence and the lower line contains the sequence of gene 1 [3]. Bold letters are point mutations compared to type I truncated mRNA; bold and italic letters represent start and stop codon; oligonucleotides (Amp 1, Amp 2, Amp 5 and Amp 8) used as primers in PCR reactions are underlined; the unspliced 81 b intron is dotted underlined.

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  1 MTSEFFARQLRAQISDDTTHPISYYKPEFYTPVDGGTAHLSVVAEDGSAV      50
    MTSEFFAAQLRAQISDDTTHPISYYKPEFYTPDDGGTAHLSVVAEDGSAV

 51 SATSTINLYFGSKVRSPVSGILFNDEMDDFSSPNITNEFGVPPSPANFIQ     100
    SATSTINLYFGSKVRSPVSGILFNNEMDDFSSPSITNEFGVPPSPANFIQ

101 PGKQPLSSMCPTIMVGQDGQVRMVVGAAGGTQITTATALICVTPFLPGRA     150
    PGKQPLSSMCPTIMVGQDGQVRMVVGAAGGTQITTATAL----------

151 HPAQPPSHADHTPMPQAIIYNLWFGYDVKRAVEEPRLHNQLLPNVTTVER     200
    ----------------AIIYNLWFGYDVKRAVEEPRLHNQLLPNVTTVER

201 NIDQAVTAALETRHHHTQIASTFIAVVQAIVRTAGGWAAASDSRKGGVPA     250
    NIDQAVTAALETRHHHTQIASTFIAVVQAIVRTAGGWAAASDSRKGGEPA

251 TECSPGGQG*
    GY*
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Fig. 3. Amino-acid sequence comparision between type I truncated encoded peptide and the gene 1 putative peptide. The amino acid sequences are represented in single-letter code. The upper line contains the peptide derived from type III cDNA sequence and the lower line contains the sequence of the protein encode by type I truncated mRNA. Bold letters represent point mutations in type III putative peptide. Italic letters represent the amino acids encoded by the unspliced intron. The asterisk represents a stop codon.

this cDNA contains the unspliced 81 bp intronic sequences, while all other introns seem to be correctly spliced.

5′ RACE was performed using Amp 5 as 3′ specific primer to clone the 5′ end of gene 1 mRNA from human placenta. PCR reaction products were cloned in pCRII vector and individual clones were sequenced. Two clones were found to contain 340 bp long inserts whose sequence was also identical to the genomic sequence (Fig. 2). As the clones did not reach the *Bgl*II site present in GGT cDNAs and in the gene sequences, we performed 5′ RACE using Amp 8 as specific primer in order to identify the 5′ end of the type III mRNA. Three individual clones were found to contain the same 84 bp insert which was perfectly identical to the corresponding gene 1 genomic sequence (Fig. 2). As compared to the truncated group II^{wt} mRNAs described by Wetmore et al. [9], type III mRNAs exhibit the sequences corresponding to the region named C and the two bases of the 3′ end of the region named B [9]. Further, three point mutations distinguish type III mRNA from group II^{wt} mRNAs. At position 44 on type III mRNA, a T replaces the C (position +9 on truncated mRNAs), and codon 8 **GCC** is replaced by **CGC** in type III mRNA.

The sequence of the type III GGT mRNA presents only one possible open reading frame (ORF) beginning with an ATG at position 36 and continuing to position 812. It encodes a 260 amino acid polypeptide which sequence reveals: (i) 7 point mutations at positions 8 (Ala → Arg), 34 (Asp → Val), 75 (Asn → Asp), 84 (Ser → Asn), 248 (Glu → Val), 251 (Gly → Thr) and 252 (Tyr → Glu); (ii) a 27 amino acid insertion due to the unspliced intron; (iii) a later stop codon due to a point mutation that suppresses the first TGA and produces an addition of 7 amino acids (Fig. 3). Because of the presence of the unspliced intron and point mutations, this polypeptide has about 80% homology with the amino acid sequence deduced from wild-type truncated mRNA peptide [9].

3.3. GGT type I and type III mRNAs expression in haematopoietic cells

The detection of the type I and type III GGT specific mRNAs in patients suffering from leukemia was achieved using RT-PCR with two set primers Amp 1-Amp 2 for type I and Amp 1-Amp 5 for type III.

As shown in Table 1, GGT type I transcripts were observed in samples of healthy subjects and patients as well as in the cell lines HL60, U937 and K562. In contrast, the product corresponding to type III mRNA was found in neither lymphocytes of healthy subjects nor in the haematopoietic cell lines. However, the product corresponding to this type of mRNA was found in 48% of the ALL subjects.

4. Discussion

Previous investigations have established the presence of at least seven genes for human GGT [3] while in rat and mouse the enzyme is encoded by a single gene [19]. From the seven genes, at least five are actively transcribed in various tissues but only the structures of two distinct mRNAs have been described.

The GGT cDNA we cloned corresponds to the gene 1 transcript previously described [3]. In general, it presents the same open reading frame (ORF) as the lung truncated mRNAs but differs by the addition of 81 bp due to the unspliced intron and several point mutations as described in Section 3.

The sequence of gene 1 upstream from the last cDNA base is identical to the 5′UTR of the truncated lung GGT mRNAs and to an intron in the GGT gene 6 (not shown). As all these mRNAs do not exceed 1.2 kb, we conclude that they start within this intron whether or not they are encoded by truncated genes. It is not clear if they are transcribed by a specific promoter or whether they represent specifically processed transcripts of the entire GGT precursor mRNA.

The amino-terminus of the precursor part of the putative polypeptide does not contain any particular address sequence nor does it have any structural homology with the amino-terminal anchoring peptide of the whole GGT molecule. As the cleavage of the GGT precursor takes place either in the endoplasmic reticulum or in the plasma membrane [20], it is possible that the truncated peptide is not cleaved.

The small subunit of the GGT contains the γ-glutamyl donor site but both subunits are required for activity [21]. Only

Table 1
Type I and type III GGT mRNA expression in hematopoietic cells

No.	Lymphocytes		Pathology	State of treatment
	Type I[a]	Type III[b]		
P1	+	–	ALL	diagnosis[c]
P2	+	–	ALL	bone narrow relapse
P3	+	–	ALL	diagnosis[c]
P4	+	–	ALL	bone narrow relapse
P5	+	–	ALL	diagnosis[c]
P6	+	+	ALL	diagnosis[c]
P7	+	+	ALL	bone narrow relapse
P8	+	+	ALL	diagnosis[c]
P9	+	+	ALL	diagnosis[c]
P10	ND	–	ALL	remission
P11	+	–	ALL	bone narrow relapse
P12	+	–	ALL	diagnosis[c]
P13	+	+	ALL	diagnosis[c]
P14	–	+	ALL	diagnosis[c]
P15	–	–	ALL	diagnosis[c]
P16	+	–	ALL	diagnosis[c]
P17	+	+	ALL	bone narrow relapse
T1	+	–		
T2	+	–		
T3	+	–		
T4	+	–		
T5	+	–		
T6	+	–		
T7	+	–		
T8	+	–		
T9	+	–		
T10	+	–		
Cell line				
HL60	+	–		
K562	+	–		
U937	+	–		

+ and – represent, respectively, the presence or absence of PCR products.
P1–P17 are RNAs prepared from ALL patient hematopoietic cells; T1–T10 are RNAs prepared from blood cells of healthy donors.
[a]Type I represents Amp 1-Amp 2 PCR reactions.
[b]Type III represents Amp 1-Amp 5 PCR reactions.
[c]Blood samples were collected before any tratment during the first hospitalization of the subject.

very early investigations have postulated that the small subunit alone could have a proteolytic activity [2]. Thus, it seems that the translation products of type III mRNA and of the other truncated mRNAs do not exhibit any GGT related activity. Furthermore, it is unlikely that these polypeptides could combine with the GGT heavy subunit to form an active enzyme.

Truncated GGT mRNAs have been identified in lung and the type III mRNA in placenta and sigmoid tissues. Surprisingly, we found this type of mRNA in about 50% of samples from ALL patients suggesting that it could represent a specific marker for this disease. As GGT genes are located near the *bcr* genes, type III mRNA pathology-specific transcription could be the consequence of the chromosomal breaks and rearrangements affecting this region, notably in the case of the ALL-associated *Philadelphia* chromosome [5]. However, our results do not allow us to correlate the type III mRNA expression either with ALL state or *bcr-abl* rearrangement. As the physiological function of this mRNA is not clear, further studies including specific antibody detection of translation products are in progress.

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