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Identification of acute myeloid leukemia patients with diminished expression of CD13 myeloid transcripts by competitive reverse transcription polymerase chain reaction (RT-PCR)

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Abstract

Normal myeloid cells of monocytic and granulocytic origin express the metallopeptidase cluster of differentiation 13 (CD13) on the surface just as leukemic blasts in most acute myeloid leukemias (AML). A minor percentage of AML patients, however, lack the surface expression of CD13 antigen. To study this difference in CD13 surface expression, specific CD13 mRNA from 44 individuals were quantified by competitive reverse transcription polymerase chain reaction (RT-PCR). Absolute values for CD13 transcripts were normalised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels to control for variations in sample preparation and mRNA degradation. By correlating normalised CD13 transcript levels and CD13 surface expression, a subgroup of AML patients was identified, having simultaneous diminished levels of myeloid CD13 transcripts and surface expression of the corresponding antigen. For this subgroup we suggest CD13/aminopeptidase N (APN) gene expression to be restricted primarily by limited amounts of transcripts. For the majority of AML patients determinants in addition to transcript levels must be involved in regulating CD13/APN gene expression. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: CD13; Acute myeloid leukemia (AML); Bone marrow; Transcript quantitation; Gene expression

1. Introduction

The glycoprotein cluster of differentiation 13 (CD13) is structurally identical to aminopeptidase N (APN) [1,2]. Within the hematopoietic system CD13 antigen is expressed on the surface of early committed progenitors of granulocytes and monocytes (CFU-GM) and more mature cells of these lineages [3,4]. Antibodies specific for CD13 do not bind to normal B or T lymphocytes. Outside the hematopoietic system CD13 expression has been demonstrated on cells and tissues including fibroblasts, bone marrow stroma cells, osteoclasts, endothelial

cells, intestinal epithelium, renal tubular epithelium and in synaptic membranes of the central nervous system [5,6].

Within the intestinal system, CD13 catalyses the removal of NH₂-terminal amino acids from peptides and it is known that it serves as a receptor for coronaviruses [7]. The function of CD13 located outside the hematopoietic system is not known but has been suggested to involve degradation of bioactive molecules [8], as well as tumour invasion and matrix degradation [9,10]. Recently, it has been shown that CD13 is possibly involved in processing and presentation of antigens [11], and it is thought to have specific roles in the control of growth and cell differentiation within both the hematopoietic and epithelial cell systems [8].

Leukemic blasts express CD13 on the surface in between 66 and 82% of acute myeloid leukemias (AMLs) [12–15], and CD13 is therefore used as one of the routine markers in diagnosis of AML. Since neither

Abbreviations: AML, acute myeloid leukemia; APN, aminopeptidase N; CD, cluster of differentiation; CFU-GM, colony-forming units for granulocytes and mononuclear phagocytes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIS, homologous internal standard; RT-PCR, reverse transcriptase polymerase chain reaction.

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function nor mechanisms regulating lineage-restricted

expression of CD13 are fully elucidated for myeloid cells, we became interested in comparing AML cases being CD13 surface positive and negative, respectively, to gain further insight into how expression of CD13 is regulated in AML.

Presence of CD13 on leukemic cells has been proposed as a potential marker to predict outcome in adult AML because CD13 positive cases have been reported to enter complete remission at lower frequency or having reduced survival when compared with CD13 negative cases [16,17]. Additional characteristics of AML patients with varying degrees of CD13 antigen surface expression could therefore be of interest.

In this study we determine surface expression and transcript levels of CD13 within mononuclear cells originating from 40 AML patients with median blast percent of 90% and four healthy controls. We relate CD13 transcript levels with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript levels in order to normalise the content of CD13 mRNA for variation in sample degradation and handling. Observed normalised CD13 transcript levels are correlated to CD13 surface expression and various clinical, pathological, and cytogenetic findings.

2. Materials and methods

2.1. Construction of homologous internal standards (HIS) for CD13 and GAPDH

RNA was extracted from mononuclear cells from an AML patient, reverse transcribed to cDNA and PCR amplified using specific primers for CD13 and GAPDH. The PCR products were sequence verified using automated cycle sequencing with dyedeoxy nucleotide terminators (373A DNA sequencer; Applied Biosystems Inc. CA, USA). Primer sequences were as follows: Sense for CD13 5'-XCGAGATCTCGAGTCCAGGGTCCAGGTTCCAG; X denotes biotin and underlined nucleotides a *Bgl*II restriction site (primer # 1 in Fig. 1A). Anti-sense for CD13 5'-CGC-AAGCTTGCGTGACAGTGCATGATTGTGCAC, where underlined nucleotides denote a *Hind*III restriction site (primer # 4 in Fig. 1A). For GAPDH sense 5'-XCGGAATTCCGGAGTCAACGGATTTGGTTCG, where X denotes biotin and underlined nucleotides an *Eco*RI restriction site. Anti-sense GAPDH 5'-CGCG-GATCCCGAGGGATCTCGTCTCTGGAAG, where underlined nucleotides denotes a *Bam*HI restriction site. All oligonucleotides were purchased from DNA Technology, Aarhus, Denmark. CD13 primers were designed to span a *Mlu*NI restriction site whereas GAPDH harboured an internal *Nco*I restriction site. Digestion with this internal restriction enzyme divided the PCR products into two (Fig. 1A). Dynabead

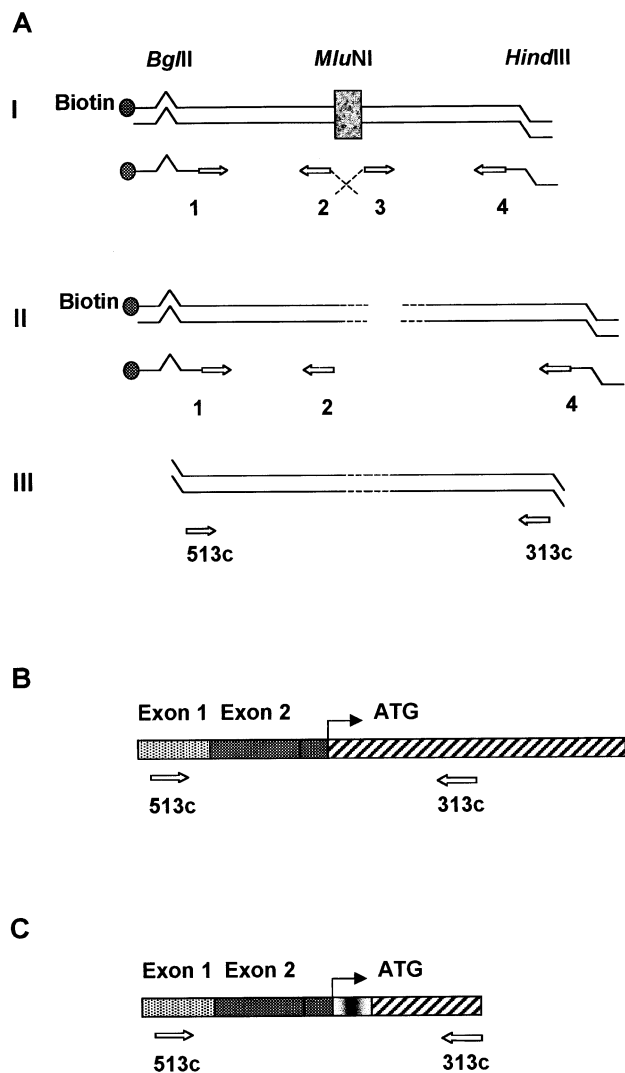






Fig. 1. Construction of homologous internal standard (HIS) for CD13 and PCR amplified areas of both HIS and target. (A) A biotinylated 5' primer with a *Bgl*II restriction site (primer # 1) and a 3' primer with a *Hind*III restriction site (primer # 4) amplified an area of CD13 containing a *Mlu*NI restriction site. *Mlu*NI digestion and biotin mediated separation of the two fragments was followed by PCR modification of 3' and 5' ends, respectively, with non-human sequences originating from linker sequences of primer 2 and 3 (I). Complementary linker sequences of primer 2 and 3 were ligated together in a PCR amplification utilising primer 1 and 4 (II). A clonable fragment was obtained by subsequent restriction digestion with *Hind*III and *Bgl*II (III). (B) Localisation of competition primers 513c and 313c on the CD13 target mRNA. Only mRNA originating from the myeloid promoter was amplified since 513c was positioned in exon 1. (C) Localisation of competition primers 513c and 313c on the CD13 HIS mRNA. 80 nucleotides of non-human origin were introduced into the exogenous transcripts.

-  Nontranslated sequences in myeloid/fibroblast cells.
-  Nontranslated sequences in intestine and myeloid/fibroblast cells.
-  Coding sequences in both intestine and myeloid fibroblast cells.
-  Non-human sequences.

(Dynal, Oslo, Norway) purification of the two fragments was followed by a PCR mediated modification of the two fragments: Linker sequences originating from cDNA of a plant transcription factor (Pallisgaard, unpublished manuscript) were added to the 5' end and 3' ends of the two fragments, respectively. Primers were as follows: Sense CD13 5'-CACCGTCCTGCAGATC-GGCAAGGCACAAAAGAGGTTGCAATTGCAAAACCAAGGGCTTCTATATTTCC (primer # 3 in Fig. 1A), anti-sense CD13 5'-CCGATCTGCAGGAC-GGTGTTGTCTAGATTTTCATCATCCATATGTG-AACCATGGTGATGGTGGGG (primer # 2 in Fig. 1A). Sense GAPDH 5'-CACCGTCCCAGATCGGC-CAGGCACAAAAGAGGTTGCAACATGGCACCG-TCAAGGCTG, anti-sense for GAPDH 5'-CCGATC-TGCAGGACGGTGTGTTGTCTAGATTTTCATCATC-CGAATTTGCCATGGGTGGAATC. Underlined nucleotides denote overlapping sequences. The linker sequences were complementary for 18 nucleotides and could hence be ligated together afterwards in a PCR as shown in Fig. 1A. Thus, non-human sequences were introduced into the middle of the target areas of CD13 and GAPDH. While attached to dynabeads modified CD13 products were digested with *Hind*III whereas GAPDH products were treated with *Bam*HI. The cut off nucleotides were removed by washing the dynabeads. Digestion of CD13 products with *Bgl*II and GAPDH with *Eco*RI ensured that all PCR fragments found in solution were ready for ligation into an already *Eco*RI-*Bam*HI or *Hind*III-*Bgl*II digested cloning and in vitro transcription plasmid pSP72 (Promega, WI, USA). The constructed plasmids were amplified by electrotransformation of the *E. coli* strain K803. Due to an ampicillin gene in pSP72, selection of ampicillin resistant colonies was possible. The cloned plasmids were sequenced to ensure the right sequences for HIS.

2.2. In vitro transcription

Linearised cloned plasmids were used as templates for in vitro transcription by T7 Polymerase following the directions of the manufacturer (Ambion kit, TX, USA). Synthesised single stranded RNA was separated from ribonucleotides and short transcripts on 5% denaturing polyacrylamide gels and HIS RNA was purified from polyacrylamide gels by phenol/chloroform extraction followed by ammonium-acetate and ethanol precipitation. HIS RNA was resolved in Tris/EDTA buffer made with diethyl pyrocarbonate treated water, the concentration was measured by absorption at 260 nm at three dilutions and HIS RNA was aliquoted and stored at -80°C in Tris/EDTA buffer containing RNasin (Pharmacia, Uppsala, Sweden).

2.3. Cell preparation, RNA extraction, and cDNA synthesis

Mononuclear cells were prepared from bone marrow aspirations by density gradient separation using Lymphoprep (Nycomed, Oslo, Norway). Mononuclear cells were cryopreserved in 10% dimethylsulfoxide and 10% fetal calf serum in liquid nitrogen. Prior to RNA extraction thawed mononuclear cells were washed 3 times in HBSS-buffer (Hanks Balanced Salt Solution, GIBCO BRL, NY, USA). Total RNA was prepared using a procedure with guanidine thiocyanate, phenol and chloroform (Genosys kit, London, UK). The RNA concentration was determined by absorption at 260 nm. cDNA synthesis was carried out with a First-strand synthesis Kit (Pharmacia, Uppsala, Sweden) by following the directions of the manufacturer; first-strand synthesis was primed by a random hexadeoxynucleotides primer provided in the kit. For each patient, seven samples were prepared with 0.5 μg patient RNA and serial diluted HIS RNAs for CD13 and GAPDH covering the range from 6×10^{10} to 6×10^4 RNA molecules.

2.4. Competitive PCR

A volume of 5 μl of $5 \times$ diluted cDNA mixture was used as template in PCR amplifications. PCR was performed on a Perkin Elmer 9600 under the following conditions: Initially 95°C for 15 min to activate the AmpliTAQ Gold (Perkin Elmer-Roche, CA, USA), then 30 repeating cycles: 95°C for 30 s, 61°C for 30 s, and 72°C for 60 s. Finally, the last extension step was 72°C for 10 min. For 25 μl PCR 0.15 u AmpliTAQ Gold was used in addition with 0.25 mM dNTP, 1.5 mM MgCl_2 , 20 pmol primers; sense CD13, 5'-GTCC-AGGGTCCAGGTTCCAG (primer # 513c in Fig. 1A) and anti-sense CD13, 5'-TGACAGTGCATGATTGTGCAC (primer # 313c in Fig. 1A). Sense GAPDH 5'-CGGAGTCAACGGATTTGGTTCG, and anti-sense GAPDH 5'-AGGGATCTCGCTCCTGGAAG. The competitive PCRs for CD13 and GAPDH were performed in separate tubes; i.e. seven PCR tubes for CD13 and seven PCR tubes for GAPDH. Template cDNA used in the seven different dilution steps of both CD13 and GAPDH originated from identical reactions. Since PCR primers for CD13 and GAPDH had similar temperature optima PCR amplifications of CD13 and GAPDH were performed simultaneously. Fig. 1.B shows the position of primer 513c and 313c on the CD13 target mRNA and Fig. 1C shows the position of the same primers on CD13 HIS mRNA. Ten microlitres of PCR product was loaded on an ethidium-bromide stained 2.5% agarose gel and electrophoretically separated before intensity of luminescence of target and HIS bands were measured by a charged-coupled device (CCD) camera (BioRad, CA, USA).

Table 1
Clinical and pathological characteristics of the 40 included AML patients

	Number
Median age (range)	60 (16–86)
Males	19
Females	25
Primary AML	35
Secondary AML	1
MDS to AML	2
Remission	2
Healthy controls	4
Spleen enlargement	3
Liver enlargement	4
Lymph node tumours	5
Skin infiltrations	1
FAB 1	10
FAB 2	12
FAB 4	12
FAB 5	5
FAB 6	1

Densiometric analysis identified dilution steps of competitive PCR where ratios between target and HIS shifted. For each patient competitive PCR was performed again in replica using cDNA corresponding to the three or four dilution steps surrounding the equivalence point in order to ensure reproducibility in the quantification. By calculating the ratio between HIS and target, regression analysis was made and initial amount of CD13 target within the samples were found and related to GAPDH level within the same sample.

2.5. Flow cytometry

Purified mononuclear cells of the bone marrow of AML patients was analysed by Coulter Profile- or Coulter Epics flow cytometry instruments (Coulter, Luton, UK). Using forward-lightscatter gating at least 10 000 cells were analysed using the log amplification mode, with negative controls being mononuclear cells labelled with second antibody layer alone as previously described [18]. Gating was made only to exclude debris and cell fragments; the percentages reported are percent positive of the total mononuclear cell number. Median blast percent of the 40 analysed AMLs was 90% ranging from 30 to 95%. The monoclonal antibody used to identify surface CD13 was My7 (Fluorescein isothiocyanate-conjugated, Coulter Immunology, Luton, UK).

2.6. Cytogenetics

Bone marrow cells were cultured *in vitro* for 30 h before metaphase chromosomes were stained with Wright's stain thereby visualising Giemsa bands [19]. The karyotypes were sorted into three prognostic groups according to the criteria laid out by Grimwade

et al. [20]. Patients with $t(8;21)$, $t(15;17)$, or $inv(16)$ were classified as belonging to the favourable group. Those with complex abnormal karyotypes (at least three aberrations in the karyotype), -5 , $del(5q)$, -7 , or abnormalities of 3q constituted the group associated with a poor prognosis. Patients with normal karyotype or aberration(s) not listed in the other two groups belonged to the intermediate group.

2.7. Patients

This study was based on bone marrow aspirations from 40 AML patients and four healthy controls. Diagnosis of AML was based on FAB criteria, immunophenotyping, and cytogenetics. At the time of diagnosis the median age was 60 years (16–84). Samples from 25 females and 19 males were analysed. Most of the patients had primary AML (35), while only one was secondary AML and two were AML developed from myelodysplastic syndrome. Two patients were in remission. Table 1 summarises other affected clinical parameters. One patient had enlarged spleen, liver, and lymph nodes, another patient had enlarged spleen and lymph nodes and a third had enlarged liver, and lymph nodes. Thus, nine patients had 13 clinical implications. Identification of FAB subtypes was based on morphological and cytochemical analysis [21].

3. Results

3.1. Validation of competitive PCR

In order to test whether amplification efficiency of HIS for CD13 and GAPDH were identical to CD13 and GAPDH target, respectively, PCR was performed using cDNA containing HIS and target at equivalent concentrations. After 24 to 40 PCR cycles, amplified product was analysed by electrophoresis and band intensities measured. Due to difference in size of HIS and target, band intensities were corrected before calculation of ratio between HIS and target for CD13 and GAPDH. The ratios were shown to be constant in the range from 24 to 40 PCR cycles (Fig. 2A). In addition, the experiments showed that the plateau for HIS and target were alike since accumulation of PCR products did not increase after cycle number 30 (data not shown).

When amplification efficiencies are identical for HIS and target, a graphic representation of the logarithm of HIS/target against the logarithm of the initial amount of HIS will be a linear curve [22,23]. For CD13 and GAPDH the HIS/target ratio was linear within the dilution range where both HIS and target were PCR amplified (data not shown). However, when either HIS or target was present in large excess only one product was amplified.

Since efficiency in cDNA synthesis and PCR may not be constant, an estimation of the reproducibility of our competitive RT-PCR assay was performed. The analysis showed that the conversion of RNA into a detectable PCR product may introduce a 1.93 ± 0.25 fold variation in the determination of initial amounts of CD13 and GAPDH transcripts (mean \pm 95% confidence limits).

The sensitivity of the competitive RT-PCR was tested by mixing various amounts of CD13 positive HL-60 promyelocytic leukemic cells with CD13 negative Nalm-6 acute lymphoblastic pre-B cells. The mixing ratios were confirmed by flow cytometry. Detection of

PCR products in dilution step corresponding to 6×10^7 RNA HIS CD13 molecules was easily observed when 1% of the cells was HL-60 whereas no target was amplified using mRNA originating from CD13 negative Nalm-6 cells (data not shown). Since flow cytometry is not reliable to define lesser percentages than 1%, replicas of two were analysed when investigating mixing ratios below 1%. When 10 HL-60 cells were mixed with 1×10^6 Nalm-6 cells, CD13 transcripts corresponding to 6×10^4 HIS transcripts were detectable (Fig. 2B) and ratios of standard and target around the equivalence point of 7.2×10^5 transcripts was linear, indicating a reliable quantification (data not shown). Thus, 6×10^4 CD13 transcripts could be identified and still be within the linear range of the method.

To adjust for differences in sample preparation and RNA degradation the amount of CD13 transcripts was normalised with GAPDH transcript amount within the same samples. GAPDH is a housekeeping gene that others have used for normalisation [24–26] and Fig. 2.C shows that GAPDH transcript levels are independent of CD13 expression on the surface.

All quantified CD13 mRNA originated from the myeloid promoter since upstream primer 513c was located in exon 1 as seen in Fig. 1B. Exon structure and sequences were defined by Shapiro et al. [27]. Thus, alternative transcription initiation from the intestinal promoter was not included in the normalised CD13 transcript level determination. Occurrence of PCR products in low dilution ranges did not originate from DNA contamination of HIS RNA since direct PCR was performed on HIS RNA preparations and did not result in any amplification (data not shown).

3.2. Transcript levels of CD13

Mononuclear cells of bone marrow aspirations from 40 AML patients and four healthy controls were analysed by competitive RT-PCR, and content of CD13 and GAPDH transcripts were determined. The resulting normalised CD13 transcript levels among patients varied from 1.1×10^{-4} to 8.9×10^{-2} , thus covering a range of more than 800 fold. Healthy controls had values between 1.4×10^{-2} and 5.1×10^{-2} and variation among them was less than four fold. The distribution of normalised CD13 transcripts is shown in Fig. 3. The normalised CD13 transcript levels fell into two groups with most AML patients having intermediate to high values whereas only a few had low values. Since the average content of GAPDH and CD13 transcripts for the majority of patients (32/40) were 3.6×10^9 and 9.3×10^7 , respectively, and the corresponding values for the remaining eight patients were 3.5×10^9 and 1.9×10^6 , the small group was characterised by not having high values of GAPDH but lower values of CD13.

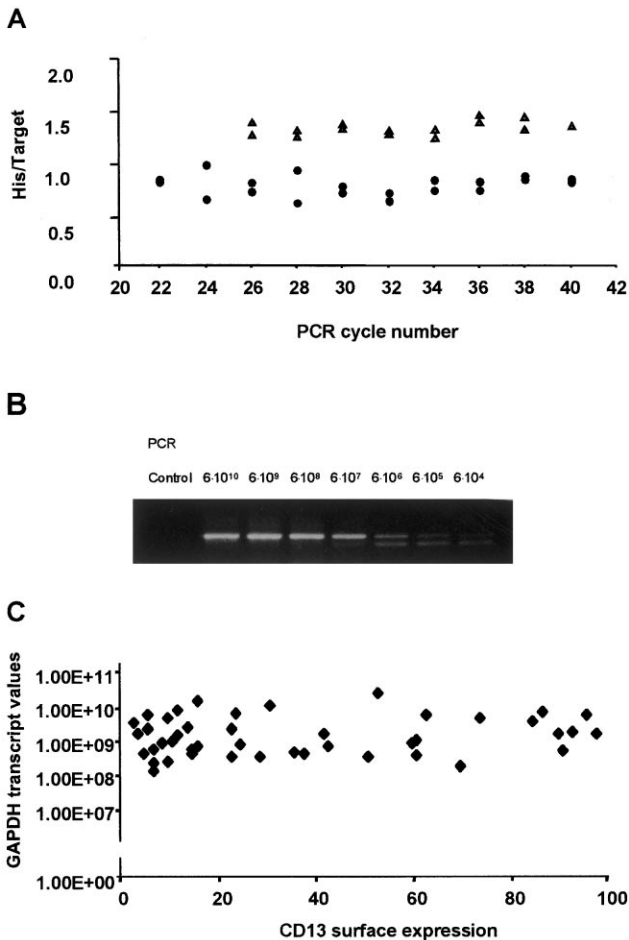


Fig. 2. Validation of competitive RT-PCR. (A) Identical amplification efficiency of HIS and target for CD13 and GAPDH. Ratio between HIS and target remains constant when competitive RT-PCR is performed on cDNA with approximately equivalent concentrations of HIS and target for both CD13 (Δ) and GAPDH (\bullet). (B) Detection of CD13 transcripts originating from 10 CD13 antigen positive HL-60 cells in a pool of 1×10^6 CD13 antigen negative Nalm-6 cells. Lane 1, PCR control where no HIS or target RNA has been added. Lanes 2–8, serial diluted CD13 HIS ranging from 6×10^{10} to 6×10^4 RNA molecules and a constant amount of cell line RNA of 0.5 μ g. CD13 target RNA corresponding to 6×10^4 HIS transcripts is detectable. (C) GAPDH transcript values and CD13 surface expression. No systematic under- or over stimulated GAPDH expression is observed with increasing surface expression of CD13.

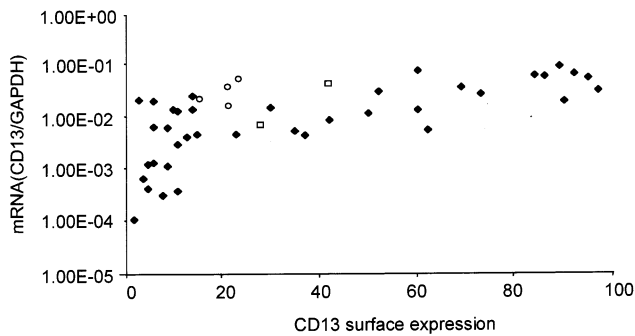


Fig. 3. Normalised CD13 transcript levels and CD13 surface expression. A general correlation between normalised transcript levels and surface expression is observed but with enhanced diversity for AML patients expressing less than 15% CD13 on the surface of their mononuclear cells. Healthy controls (○), AML patients in remission (□) and AML patients (◆).

3.3. Immunophenotyping

Mononuclear cells were analysed by flow cytometry, and expression of myeloid CD13 antigen on the surface was determined. This was done on the same patient samples as those used for determining normalised CD13 transcript levels by competitive RT-PCR. Flow cytometry was carried out prior to cryopreservation. The relation between cell surface expressed CD13 and normalised CD13 transcripts is shown in Fig. 3.

For the 32 AML patients and the four healthy controls having intermediate to high values of normalised CD13 transcripts levels there was a linear correlation between normalised CD13 transcript levels and cell surface expression of CD13 ($r = 0.59$, $P < 0.001$, $n = 36$). The correlation was, however, ambiguous since $r^2 = 0.35$, and r^2 is a measure of the proportion of explained variance of surface expressed CD13 which can be explained by variation in normalised CD13 transcripts. The limited correlation was also reflected in similar transcript levels mediating surface expression as different as 6 or 60% positive mononuclear cells or similar transcript levels resulting in 22 or 90% positive mononuclear cells.

As shown in Fig. 3, healthy controls had higher normalised transcript levels than patients with similar CD13 surface expression. The ten patients with surface expression below 15% and intermediate to high normalised CD13 transcript levels were defined as CD13^{high}.

For patients having low normalised CD13 transcript levels another pattern emerged. The eight patients had simultaneous low values of normalised CD13 transcripts and low expression of CD13 antigen on the surface of mononuclear cells. None of them had more than 15% myeloid CD13 antigen on the cell surface and they were defined as CD13^{low}.

Intensities of CD13 surface antigen were registered as average fluorescence and divided into three logarithmic decades defined as low, intermediate, and high fluorescence. Since all analyses were performed on a flow cytometer that was regularly tested and trimmed to avoid day to day variations, comparison of CD13 antigen intensities of the AML patients was possible. Of 44 samples analysed 34 had low, and ten had intermediate CD13 antigen intensities. For patients of both CD13^{high} and CD13^{low} only low average fluorescence of CD13 surface intensities were observed. Concentrations of CD13 on mononuclear cells were therefore considered to be comparable for the ten patients of CD13^{high} and the eight patients of CD13^{low}. For the ten patients with intermediate CD13 antigen intensities, seven had surface expression of CD13 antigen on more than 80% of the mononuclear cells (data not shown). Thus, patients with high percentages of CD13 positive mononuclear cells may have slightly increased concentrations of CD13 antigens on the cellular surfaces. However, none of the ten patients with intermediate CD13 antigen intensities had significantly increased normalised CD13 transcript levels as compared with the remaining patients as shown in Fig. 3.

During myeloid differentiation expression patterns of myeloid CD markers gradually change so maturation of myeloid cells of AML patients can be judged by immunophenotyping of CD13, CD14, CD33, and HLA-DR. The expression of CD33 surface antigen was in average 72% (2–99%) for all 40 patients. CD13^{low} had an average of 72% while CD13^{high} had an average of 61% (Table 2). A similar pattern was observed CD14 antigens. The average was 16% (1–71%) for all 40 patients with while CD13^{low} and CD13^{high} had average values of 17 and 24%, respectively. HLA-DR was found on mononuclear cells in an average of 50% (2–90%) for all 40 patients, while CD13^{low} had an average of 52% and CD13^{high} had an average of 36%. Thus, CD13^{low} as compared with CD13^{high} had similar expression rates of the myeloid immature markers CD33 and HLA-DR, as well as the mature marker CD14.

3.4. Cytogenetics and FAB classification

At the time of diagnosis a chromosome study was carried out in 33/40 patients. The karyotypes were grouped into the three prognostic groups defined by Grimwade et al. [20]. (See Section 2). None of the patients belonged to the prognostically favourable group. Three had complex abnormal karyotypes (1 associated with 5q- and -7). The remaining 30 belonged to the intermediate group. Nineteen presented a normal karyotype.

All seven cytogenetically analysed patients in CD13^{low} had intermediate prognoses (Table 2). For

patients in CD13^{high} a comparable distribution was observed with 6 having intermediate prognoses, one having poor prognosis and one was not cytogenetically examined.

Classification of the 40 patients into FAB categories is summarised in Table 1. For the whole range of observed normalised CD13 transcript levels including CD13^{low} and CD13^{high} no obvious correlation to FAB classification was found.

3.5. Clinical parameters

For the 40 AML patients median age at diagnosis was 60 years. Four are still alive 5 years after diagnosis, two are alive 4 years after and one is alive 1 year after diagnosis while 33 patients are dead. In survival analysis the 10/10–1997 was used as censor date. The overall average of survival was 750 days (1–3982) while it was 467 days (1–3982) for those who died.

For CD13^{low} patients median age at time of diagnosis was 60 years and average survival was 627 days (1–3126) (Table 2). 168 days after diagnosis 50% were still

Table 2

Clinical, pathological, cytogenetical, immunophenotypic, and molecular characteristics of CD13^{high} and CD13^{low}

	CD13 ^{high}	CD13 ^{low}
<i>Clinical parameters</i>		
Median age (range)	68 (44–85)	60 (28–86)
Average survival in days	361	627
Blast population in BM (%)	85	81
Spleen enlargement	2	1
Liver enlargement	2	2
Lymph node tumours	4	1
Skin infiltrations	0	1
Leukocytes ($\times 10^9/l$)	121	168
Thrombocytes ($\times 10^9/l$)	52	98
Haemoglobin (mmol/l)	6	6
<i>Pathological parameters</i>		
FAB 1	2	2
FAB 2	2	1
FAB 4	4	3
FAB 5	2	2
<i>Chromosomal parameters</i>		
Favourable prognosis	0	0
Intermediate prognosis	6	7
Poor prognosis	1	0
Not cytogenetically examined	3	1
<i>Immunophenotypic parameters</i>		
Average CD 14% positive cells	24	17
Average HLA-DR% positive cells	36	52
Average CD 33% positive cells	61	72
<i>Molecular parameters</i>		
Average content of total GAPDH mRNA	2.5×10^9	3.5×10^9
Average content of total CD 13 mRNA	1.7×10^7	1.9×10^6

alive. For CD13^{high} patients median age at diagnosis was 68 years and average survival was 361 days (2–2624) and 49 days after diagnosis 50% were alive. One survivor was found within CD13^{high} while none of the patients in CD13^{low} is still alive. Thus, CD13^{low} patients tended to survive longer than CD13^{high} patients but the difference was not statistically significant.

In order to leave age at diagnosis out of account when comparing survival between CD13^{low} and CD13^{high} the seven patients with the highest normalised CD13 transcript levels and a median age of 69 years were compared with the five patients with the lowest normalised CD13 transcript levels and a median age of 70 years. Thereby the difference in survival rate became more pronounced since the seven high patients survived 107 days in average while the five low patients survived 789 days ($P = 0.075$). The seven high and the five low had comparable distribution regarding prognostic groups.

Regarding spleen and liver enlargements, lymph tumours and skin infiltrations these complications were not restricted to any particular group of patients but found randomly among all 40. Average concentration of leukocytes was for all 40 patients $92 \times 10^9/l$ while CD13^{high} had $121 \times 10^9/l$ and CD13^{low} had $168 \times 10^9/l$ leukocytes. Thrombocyte concentrations were in average for all 40 patients $66 \times 10^9/l$, whereas CD13^{high} and CD13^{low} contained $52 \times 10^9/l$ and $98 \times 10^9/l$, respectively. Haemoglobin content was 6 mmol/l in average for all patients, just as it was 6 mmol/ for both CD13^{high} and CD13^{low}. Thus, no connection between normalised CD13 transcripts and leukocytes, thrombocytes or haemoglobin was found.

4. Discussion

At our department 8% of 743 de novo AML patients had surface expression of CD13 on less than 15% of mononuclear cells of their bone marrow. To achieve a better understanding of why certain AML patients lack CD13 myeloid antigens, determination of normalised CD 13 transcript levels was performed on cryopreserved material.

Values of normalised CD13 transcripts fell into two groups with the majority of AML patients having intermediate to high levels and only a few with low levels. Four healthy controls had high transcript levels as compared with AML patients with similar surface expression. The heterogenous nature of their bone marrow cells could offer an explanation since it has been observed that CD13 specific mRNA can be present in the human T cell lines *HuT78* and *H9* without simultaneous expression of CD13 antigen on the surface [28,29].

For AML patients with less than 15% surface expression of CD13 on mononuclear cells, enhanced diversity of normalised CD13 transcript levels was observed. Patients from CD13^{low} and CD13^{high}, respectively, had normalised CD13 transcript levels as different as 1.1×10^{-4} and 2.1×10^{-2} but CD13 expressed on 3 and 2%, respectively, of their mononuclear cells. Such differences of up to 200-fold variation in normalised transcript levels were found only for AML patients having less than 15% surface expression of CD13 antigen. For patients with CD 13 surface antigen expression above 15%, variation of normalised CD13 transcript levels was below ten fold when surface expression was alike. Variation of normalised CD13 transcript levels within CD13^{low} and CD13^{high} was of a similar range (12 and 10 fold).

Since bone marrow cells had median blast count of 90% for both CD13^{high} and CD13^{low}, respectively, and T lymphocytes constituted only 2% of mononuclear cells for both groups, the differences in normalised transcript levels were not caused by differences in compositions of the analysed cell populations. Thus, CD13^{high} was not enriched by T lymphocytes having intracellular CD13 mRNA but lacking surface expression of the CD13 antigen [28,29].

The diversity of normalised CD13 transcript levels found for patients with less than 15% CD13 antigen on the surface of their mononuclear cells was not caused by limitations of the competitive RT-PCR method. The theoretical demands of the method were empirically tested with a satisfactory result. Hence, HIS and target had similar amplification efficiencies when concentrations of templates were close to each other. Graphical presentation of the logarithm of HIS/target was linear within the dilution range where both HIS and target amplified. Ratios of HIS to target have by others been reported to be important for obtaining reliable quantifications [30,31]. They show that only when ratios of HIS to target are close to each other are the amplification efficiencies similar and only then will the competitive RT-PCR method result in a linear relation [22,30,31]. In the present study, quantifications have only been performed on data within this range.

Similar amplification efficiencies were obtained even though the length of HIS and target varied with 79 base pairs for CD13 and 64 base pairs for GAPDH. This is in accordance with observations made by others, who showed that amplification efficiencies were unaffected by differences in length of as much as 150 bp between 2 targets [30,32].

The endogenous standard GAPDH was necessary to control for variations in sample preparation and mRNA degradation. Others have used GAPDH as internal control in PCR quantification [24–26]. However, to ensure that GAPDH gene expression was not systematically under- or over stimulated in AML pa-

tients with increasing surface expression of CD13 antigen, comparison of GAPDH mRNA levels relative to surface expression of CD13 was performed. As seen in Fig. 2.C no such influence was indicated. Furthermore, no correlation was observed between GAPDH mRNA levels and CD13/GAPDH mRNA levels (data not shown).

Sensitivity of the competitive RT-PCR method was tested by mixing cell line material being positive and negative for surface expression of CD13. Since CD13 mRNA originating from ten CD13 positive HL-60 cells was easily detected in a pool of 1×10^6 CD13 negative Nalm-6 cells the sensitivity of the assay was determined to be $10/10^6$. This is in the same order of magnitude as others have reported [33,34].

Reproducibility of competitive PCR ranges from two to ten folds [35,36]. Thus, our reproducibility of approximately two fold was considered to be acceptable.

Dependent on tissue type, transcription of the CD13/APN gene originates from two different promoters [27,37]. For leukemic B-cells, surface expression of CD13 has been suggested to be under promoter-mediated regulation since stroma cells suppress transcription from the CD13/APN gene [38]. Accordingly, studies of activation-dependent induction of CD13 in T-cells have resulted in suggestions of CD13/APN gene expression being regulated at transcriptional level by affecting promoter activity as well as at the post-transcriptional level by affecting stabilisation of CD13 mRNA [39]. Post transcriptional events affecting CD13 mRNA conditions have also been observed in rat frontal cortex [40], just as promoter mediated regulation of CD13 surface expression has been shown to be dependent of promoter activity in endometrial cells [41]. Hence, regulation of CD13/APN gene expression is complex and includes several points susceptible to influence.

In the present study, analyses were performed on limited amounts of cryopreserved patient material and did not allow for CD13/APN gene regulation investigations. Therefore, no substantial conclusions of the mechanism controlling CD13/APN gene expression could be made. However, observations of normalised CD13 transcript level and surface expression do lend support to some considerations about regulation of CD13/APN gene expression.

Simultaneous low levels of normalised CD13 transcript and CD13 surface expression were observed for CD13^{low}. Whatever caused the lack of CD13 transcript—whether it was due to suppression of transcription or degradation of mRNA—it suggests the amount of CD13 transcripts to be an important determinant of low surface antigen expression within these specific AML patients.

For the remaining 32 AML patients additional factors must be involved supported both statistically by $r^2 = 0.35$ and by observations of normalised CD13

transcripts. Intermediate to high levels of CD13 transcripts within CD13^{high} did not mediate increased surface antigen expression, as compared with CD13^{low}, and patients having similar transcript levels had different levels of surface expression. Since only mononuclear cells were immunophenotypically examined, none-cell-adherent circulating isoforms of CD13 [42], may be present in these AML patients having intermediate to high levels of normalised CD13 transcripts.

Division of AML patients having less than 15% CD13 on their mononuclear cells into CD13^{low} and CD13^{high} was based on the distribution of normalised CD13 transcript levels among the included AML patients. Conceivably no value distinctly separates the cases from each other but a gradual transition exists from low level normalised CD13 to high level normalised CD13 transcripts. The division into CD13^{low} and CD13^{high} was made to enable a judgement of possible correlations between transcript levels and other parameters. The two groups were very similar regarding myeloid maturation of their cells as judged by immunophenotyping of CD14, HLA-DR and CD33, just as their chromosome abnormalities, a known very important prognostic factor [20], were comparable.

Individuals having low transcript levels and less than 15% CD13 surface expression had a tendency to survive longer than patients having high transcript levels. However, as the number of patients included in this study was too small to justify statistical analysis, one might only speculate, whether CD13^{low} is a good risk factor in AML.

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