Incidence of preleukemic fusion genes in healthy subjects

Minireview

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The diagnostics of leukemia relies upon multi-parametric approach involving a number of different pathology disciplines such as flow cytometry, histopathology, cytogenetics and molecular genetics [fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR)]. Childhood leukemia is often determined by the presence of specific chromosomal translocation that entails the generation of preleukemic fusion genes (PFG). In the last two decades, several studies have reported observations that PFG are present in healthy population and not necessarily result in leukemia. The first such study by Limpens and colleagues on t(14/18)/ *BCL2-JH* [1] and next in line [2, 3] led to many questions regarding the significance of these chromosomal translocations in leukemogenesis. However, the data on the incidence of PFG are contradictive. This review aims to highlight the molecular genetic approaches used by various studies with regard to differences in diagnostics and incidence of PFG in healthy subjects. The focus is on the incidence and prevalence of the most common PFG such as *TEL-AML1*, *MLL-AF4*, *BCR-ABL (p190)*, *AML1-ETO*, *PML-RARA*, and *CBFB-MYH11* detected in umbilical cord blood, in neonatal blood spots (Guthrie cards (GC)), bone marrow, peripheral blood and tissues of amortized fetuses. We conclude that the incidence of PFG is significantly higher than incidence of leukemia and more sophisticated analysis of PFG in leukemogenic cell populations is warranted to relate the occurrence of PFG with leukemia. The emerging notion is that only those PFG may contribute to development of leukemia which arise in stem cells at specific time windows during development. Thus, screening of PFG in subpopulations of stem cells may be a challenge for assessment of predisposition to leukemia and for validation of cell transplant to minimize donor cell-derived leukemia.

Key words: preleukemic fusion genes, childhood leukemia, stem cells

Leukemia is a cancer characterized by abnormal proliferation of hematopoietic cells. It is the most common cancer in children representing about 30% of all childhood cancers, where acute lymphoid leukemia (ALL) at 81% is the most frequent leukemia in Europe, followed by acute myeloid leukemia (AML) with 15%, and other three, markedly rare subgroups of chronic myeloid leukemia (CML) at 1.5%, unspecified (1.3%) and other specified leukemia (<0.5%) [4]. In childhood leukemias, there is a clear evidence that largely non-overlapping subtypes of disease may be identified based on the genetic abnormalities which they acquire. Chromosomal translocations as a consequence of double-strand breaks (DSB) are the most common genetic aberrations in leukemias and one of the tumor hallmarks [5, 6]. Depending on the chromosome breakpoints, a translocation can result in formation of so-called preleukemic fusion gene (PFG) followed by a disruption or mis-regulation of the normal gene function [7, 8].

Abbreviations: ALL – acute lymphoblastic leukemia; AML – acute myeloid leukemia; BM – bone marrow; CML – chronic myeloid leukemia; DCL – donor cell-derived leukemia; FISH – fluorescent in situ hybridization; GC – Guthrie cards; HSC – hematopoietic stem cells; HSC/PC –hematopoietic stem /progenitor cells; LIC – leukemia-initiating cells; LSC – leukemia stem cells; MNC –mononuclear cells; PB –peripheral blood; PCR –polymerase chain reaction; PFG – preleukemic fusion genes; RT qPCR – real-time quantitative PCR; RT PCR – reverse transcription PCR; UCB – umbilical cord blood; WBC – white blood cells

Common chromosomal translocations in pediatric leukemia include t(12;21)/*TEL-AML1* (~25%), t(11;4)/*MLL-AF4* (~5%), t(9;21)/*BCR-ABL* (~5-10%), t(1;19)/*E2A-PBX1* (~5%) for B-lineage ALL, and t(15;17)/*PML-RARA* (15%), t(8;21)/*AML1-ETO* (4-11%), and inv(16)/*CBFB-MYH11* (8-12%) for AML. Many of them are present also in other types of leukemia and adults, e.g. 87% of patients with CML harbor *BCR-ABL* [9]. Other very frequent chromosomal aberrations include high hyperdiploidy with 51-60 chromosomes, hypodiploidy (<44 chromosomes) and rearrangements of *IgH* and *TCR* genes [10], however, these lesions will not be discussed in this review.

Cytogenetically different ALL subtypes create clinically and biologically distinct entities of disease. Distribution of ALL subtypes is uniquely divided based on age. The *TEL-AML1* ALL occurs at very high frequency between 2 and 5 years of life and almost completely absents in adults. In contrast, *MLL-AF4* ALL often appears early after the birth and the incidence of *BCR-ABL* increases with age. Importantly, specific genetic subtypes are often associated with initial treatment response, a therapy-depending risk of relapse and the overall survival rate [11, 12]. Poor prognosis is preferentially associated with chromosomal breakpoints within the *MLL* intron (e. g. *MLL-AF4*) with <50% of 5-year overall survival, despite the fact that patients receive a very intensive chemotherapy [13, 14]. *TEL-AML1* showed excellent prognosis (> 90% survival) with intensive doses of L-ascorbic acid 2-phosphate magnesium [15]. The therapeutic outcome for ALL associated with *BCR-ABL* has been improved after using tyrosine kinase inhibitors, however, it is still poor, with event free survival ~70% and a high resistance to therapy [16, 17, 18].

Epidemiological evidence has suggested that some pediatric leukemia may be initiated by the formation of PFG prenatally after exposure to external factors. Ionizing radiation, chemotherapy or genetic disorders are known risk factors for leukemia, but explain only a small fraction of cases [19, 20]. There are few lines of evidence suggesting prenatal origin of pediatric leukemia: (i) a common clonal origin of concordant leukemia in monozygotic twins *via* monochromic placental vasculature [21, 22, 23]; (ii) the presence of PFG in archived neonatal blood spots [24]; (iii) findings obtained by molecular screening of umbilical cord blood (UCB) for PFG [2]. Several scientific groups have later described the incidence of PFG in UCB of the healthy population [2, 3, 25, 26, 27]. While the data were not always consistent, most of the groups reported that the incidence of PFG-positive UCB exceeded about 100-fold the incidence of leukemia. Obviously, reliable estimation of PFG in UCB hematopoietic stem/progenitor cells (HSC/PC) with leukemogenic potential may be diagnostically important [28].

In addition to UCB samples, different sources of hematopoietic cells, such as peripheral blood (PB) from children or adults, infant bone marrow (BM), fetal BM or liver from amortized fetuses were screened. Most of the studies investigated *TEL-AML1* and *BCR-ABL* fusion genes. Other PFG were described only sporadically. In all cases, nested PCR (RT PCR) or real-time quantitative PCR (RT qPCR) was performed as a primary screening method. For the verification of positive samples, different methods on RNA or DNA levels were used, such as re-screening by RT qPCR, nested RT PCR, Southern blot, dot blot, FISH and sequencing the PCR product. Brassesco reviewed the incidence of PFG in healthy individuals in 2008 [29]. *BCR-ABL* was then partially summarized by Ismail and colleagues [30].

The observations that some of PFG are present in healthy individuals have triggered more attention due to their significance in etiology of leukemia and possible significance for the donor cell-derived leukemia (DCL) [31]. Currently, more than 50,000 patients *per* year receive hematopoietic stem cell transplantation, typically to treat malignant diseases [32]. In some rarely cases, the treatment following BM or UCB allogeneic transplantation resulted in a 'relapse' associated with DCL [31]. During forty years, 76 reports of DCL following 24 UCB and 52 BM transplantations were published in the National Library of Medicine's PubMed database [33]. In general, DCL have a poor prognosis, lower than 30%, with a median duration of survival approximately 5 months. While mechanism of DCL remains speculative, the possibility that DCL may be triggered by preleukemic clone with PFG preexisting in transplant cannot be ruled out [34].

It is obvious that molecular and genetic methods are crucially important as they help to determine and refine diagnosis, estimate prognosis, and suggest the most appropriate treatment. Determination of the incidence of PFG highly depends on the sensitivity and specificity of the screening methods, which partially differ between laboratories and between treatment protocols.

The purpose of this paper was to give an update and overview of all available data on most frequent leukemia-associated PFG (*BCR-ABL*, *MLL-AF4*, *TEL-AML1*, *AML1-ETO*, *PML-RARA* and *CBFB-MYH11*) in healthy subjects. Our review summarizes and compares all available data on the incidences of PFG in healthy individuals, and the methods. We focused on sensitivity and specificity of applied screening methods used, and sources of tested samples. For this aim, the main scientific databases including Web of Science and Scopus were searched through with relevant key words and all retrieved publications were considered.

In order to assure confidence in the results and conclusions of these studies, we have to take into consideration several important criteria, including (i) *statistical power*, ensuring sufficient number of subjects enrolled/screened in the study. For example, if the expected incidence of *TEL-AML1* in cord blood is about 1%, i.e. one positive case *per* 100 probands, the size of the sample should be at least 100, but preferably higher multiples of this number which can increase the chance of finding at statistically significant value, (ii) choice of *a correct screening method*, in terms of sensitivity, specificity and reliability. *Sensitivity* of the method quantifies its threshold for PFG detection. If the sensitivity of the screening assay is too low, we can expect to miss the real PFG values. In case when the positivity incidence is very close to the threshold of the method sensitivity, the final numbers may be

rather semi-quantitative, representing an estimate of the actual PFG incidence. *Specificity* of the screening is aimed to minimize the amount of false positives in the sample set. *Reliability* of the screening is meant in terms of the risk of false positivity due to cross-contamination, therefore, inclusion of negative controls must be an essential part of the methodology. However, some methods are inherently susceptible to cross-contamination, especially nested PCR is notoriously known for contamination problems: opening the micro-tube after 1st PCR could lead to false positive results through cross-contamination. From this point of view, the PFG incidences achieved by using two-tube nested PCR, especially when the numbers markedly stand out from the trend and could not be confirmed by other groups, cannot be considered as reliable, but they rather invoke doubts and should be excluded. In contrast, the RT qPCR with intro-

duction of non-template controls (NTC) has an extremely low risk of cross-contamination and therefore, should be regarded as a reliable method.

PFG molecular screening

BCR-ABL fusion gene. BCR-ABL is one of the most investigated PFG in healthy people. According to the breakpoint location within the BCR gene, the resulting BCR-ABL fusion protein has two variants: shorter 190-kDa protein (p190), which is more common in ALL, and longer 210-kDa (p210), which is usually presented in CML. In three studies where PB has been tested, the correlation of both variants with age has been analyzed (Table 1). As a result, BCR-ABL p210 fusion transcript has shown significant upward trend with

Table 1. Incidence of *BCR-ABL* in healthy subjects

Studies	Source	Methods	Sensitivity	*BCR-ABL* variant, positive/examined subjects (incidence, %)	Subjects, age	Analyzed sample
Biernaux (1995)	UCB	Nested PCR	Up to 10^{-8}	p210 0/22 (0%)		10^8 WBC
	PB	Nested PCR		p210 1/22 (4.5%)	Children PB: <13 years	4-10µg of total RNA for RT
				p210 22/73 (30%)	Adult PB: 20-80 years	
		Sequencing		p210 23/23 (100%)		
Bose (1998)	PB	Nested PCR	10^{-5}-10^{-6}	p190 11/16 (68%)	Adult: 23-46 years	10^7 WBC
				p210 4/15 (27%)	Adult: 23-46 years	total RNA
						40 replicates
Uckun (1998)	Fetal liver	Nested PCR	10^{-4}	0/13 (0%)	Gestational age:	Total RNA
	Fetal BM	Nested PCR	10^{-4}	0/16 (0%)	15-22 weeks	
	Neonatal BM	Nested PCR	10^{-4}	0/6 (0%)		
		Standard PCR	10^{-2}	0/35 (0%)		
Song (2011)	UCB	Nested PCR	10^{-4}	p190 21/50 (42%)		$8-16 \times 10^6$ MNC
				p210 8/50 (16%)		0.5g total RNA for RT
	PB	Nested PCR	10^{-4}	p190 4/10 (40%)	Newborns	3µl cDNA/1 round, 1µl
				p210 2/10 (20%)	Newborns	cDNA/2 round RT PCR
				p190 22/29 (75%)	Children < 25 years	
				p210 11/30 (36%)	Children < 25 years	
		RT qPCR	10^{-4}	p190 33/41 (80%)	Adults > 25 years	
				p210 21/34 (61%)	Adults > 25 years	
				p190 26/39 (67%)	Children +Newborns	
				p210 13/40 (32%)	Children +Newborns	
Boquett (2013)	PB	Nested PCR	NA	P210 2/30 (6.67%)	Adults > 40years	6µl total RNA for RT
Ismail (2014)	PB	Nested PCR	10^{-6}	p190 0/44 (0%)	Children: 2-16 years	1µg RNA to RT
				p210 4/44 (9.1%)	Children: 2-16 years	
				p190 0/145 (0%)	Adults: 20-86 years	
				p210 15/145 (10.3%)	Adults: 20-86years	
Skorvaga (2014)	UCB	RT qPCR 1	$1-3 \times 10^{-5}$	p190 50/200 (25%)		10^7 MNC
		RT qPCR 2	$1-3 \times 10^{-5}$	p190 4/15 (26.6%)		1µg total RNA to RT
				in total 6.25%		2µl cDNA *per*
		Nested PCR	$1-3 \times 10^{-5}$			reaction
		Multiplex PCR	$0.2-1 \times 10^{-3}$	p190 0/135 (0%)		
Kosik (2015)	UCB	RT qPCR 1	$1-3 \times 10^{-5}$	p190 92/500 (18.4%)		10^7 MNC
		RT qPCR 2	$1-3 \times 10^{-5}$	p190 27/90 (30%)		1µg total RNA to RT
		Sequencing		p190 20/22 (90.09%)		2µl cDNA *per* reaction
				In total 5%		

PB – peripheral blood; MNC – mononuclear cells; RT – reverse transcription; RT PCR – nested PCR; NA – non available; WBC – white blood cells

age in these studies, which recorded the following incidence values in UCB/GC of newborns, in PB of children, and in PB of adults: 0/22 (0%), 1/22 (4.5%), and 22/73 (30.1%) in first, 2/10 (20%), 11/30 (36.7%), and 21/32 (65.6%) in second, and, finally N/A, 4/44 (9.1%), and 15/145 (10.3%) in third study, respectively [30, 35, 36]. Overall, the data on the incidence of BCR-ABL p210 transcripts in PB of healthy children are very limited. The recently published results by Ismail et al. [30] show a slightly higher incidence rate (4/44; 9.1%) than that reported by Biernaux et al. (1/22; 4.5%), but much lower than the incidence recorded by Song and colleagues, namely 36.7% (11/30) [35]. In terms of sensitivity of the method, the study of Biernaux and colleagues stands up due to very high sensitivity of a novel RT PCR method allowing them to detect 1 copy of BCR-ABL p210 RNA in an equivalent quantity of 10^8 cells. The RT PCR optimization was achieved by (i) increasing the amount of total RNA in reverse transcription reaction from a standard 1 µg to up to 10µg (ii) spending entire cDNA for 1st round PCR amplification, and (iii) using 1/10 of 1st PCR for nested PCR. The authors declare the adherence to very strict conditions and controls when performing all experiments [36]. However, the amount of screened subjects, especially in the group of children, is relatively very low (n = 22), therefore, the statistical relevance of the data is limited. On the other hand, more recent study by Ismail and colleagues [30] recorded higher incidence, paradoxically using a method with two-orders lower sensitivity on the sample of 2-fold larger size (n = 44). The data of Song and colleagues [35] are questionable due to several fold higher incidence as compared to other available reports, which might likely be caused by cross-contamination of samples during nested PCR.

Relationship of BCR-ABL incidence to the gender has also been analyzed. While the study of Ismail et al. [30] indicated higher risk of p210 fusion for males at 12/98 (12.2%) against females at 7/91 (7.7%), the study by Boquet et al. has reported p210 fusion transcripts only in females (2/30, 6.7%) [37]. The p190 transcripts have not shown any correlation with age or gender [30, 35]. However, approximately a 2-fold higher incidence of p190 than p210 fusion transcripts has been detected within the study of Song et al. in children and adults, 80%:62% and 61%:32%, respectively [35]. Similar ratio between p190 (11/16) and p210 (4/15) fusion transcripts has been previously observed by Bose et al. in adults (69% and 27%, respectively). With respect to the sensitivity of the method, Bose and colleagues used a modified nested PCR assay analyzing the total amount of cDNA synthesized from 10^8 white blood cells (WBC) in 40 replicate PCR tests, achieving a relatively high sensitivity of the screening method, i.e. 10^{-5} to 10^{-6}. The two groups, Biernaux's and Bose's, achieved very similar incidence of p210 fusion transcript in healthy adults, namely 4/15 (27%) and 22/73 (30%). However, the validity of the results is limited due to small size of the screened samples. Contradictorily, this incidence has not been confirmed in the study by Ismail et al., where the p190 transcript was not detectable by nested PCR in healthy children and adults [30]. Of note, the usefulness of

the results stemming from Song's and Bose's studies for the assessment of the BCR-ABL incidence is very limited due to low statistical power and reduced reliability of the two-tube nested PCR, which was used in these studies.

In 1995, Biernaux et al. for the first time investigated BCR-ABL in UCB of healthy individuals [36]. This group failed to detect this transcript in very limited number of analyzed UCB (0/22), although using nested RT PCR approach with the highest sensitivity level recorded so far (1 x 10^{-8}). Recently, several groups extended studies on BCR-ABL in UCB. Song et al. reported a high 42% (21/50) incidence of BCR-ABL p190 and 16% (8/50) incidence of BCR-ABL p210 transcripts in UCB [35]. Again, the number of subjects (50) enrolled in this study was too low to make any conclusion. In addition, the method used (two-tube nested PCR) may have lower reliability due to very high risk of cross-contamination, even though the authors state that the 2nd round PCR was performed in a different building. Evidently, the incidence of BCR-ABL fusion transcripts in UCB as well as in other sources reported by Song et al, highly exceed the incidences estimated by all other groups. Our research group defined significantly lower incidence of BCR-ABL p190 in UCB, i.e. 6.25% on 200 subjects and later refined to 5% on 500 subjects [26, 28]. Moreover, one research group has investigated the presence of BCR-ABL transcripts in fetal BM, fetal liver and infant BM without finding any positive samples [38]. However, this study suffers from low number of screened samples, namely 13 fetal livers, 16 fetal BM and 6 infant BM. The sensitivity of their PCR derived for MLL-AF4 was significantly lower as compared to ours, achieving about 1% of MLL-AF4+ cells in 10^7 total cell number (1 x 10^{-2} sensitivity) whereas the nested PCR was 100-fold more sensitive (1 x 10^{-4} sensitivity). However, the sensitivity of the BCR-ABL assay, which may differ from the MLL-AF4 sensitivity, was not determined, thus contributing to drawbacks of this study. Similarly, the specificity of BCR-ABL analysis was limited, in contrast to that of MLL-AF4, which was remarkable and consisted of several complementing techniques.

The presence of BCR-ABL p210 positive cells in PB of healthy adults has been estimated only sporadically, ranging values from about 10^{-4} to up to 10^{-8} [35, 36, 39]. In general, these data are likely inaccurate, representing only rough estimates of actual frequencies due to low statistical power of the studies and high risk of cross-contamination of the nested PCR screening method. However, the presence of BCR-ABL fusion gene in healthy subjects was further substantiated by more recent case reports reliably showing t(9;22)/BCR-ABL p210 chromosomal translocation in asymptomatic subjects who never developed CML. One of the case reports [40] describes a 39-year old male with elevated WBC of 15,000/µl, but otherwise asymptomatic, with no evidence of any cytogenetic or molecular abnormalities, with an exception of the presence of BCR-ABL detected in 51 of 300 cells in his peripheral blood by FISH and RT qPCR. In addition, FISH for t(9;22)/BCR-ABL was tested positive in 52% of BM cells. The patient continued to

be asymptomatic at 1-year of surveillance. Another case report shows data on a 71-year old male presented with a lesion under his right eye, with WBC of 6,600/µl and a moderate increase in myeloid precursors in BM [41]. FISH analysis confirmed the findings of cytogenetics in both BM (62.5% of cells positive for *BCR-ABL*) and in PB (73.5% positivity), these data may suggest a relatively high proportion of p210+ cells present in BM/PB of an asymptomatic individual. Despite the fact that this man was asymptomatic, he underwent a treatment with imatinib and achieved a major molecular response exhibiting as a significant decrease of *BCR-ABL* copy number estimated by RT qPCR. In these two case reports, *BCR-ABL* positivity has been proved by FISH method as an essential technique for the cytogenetic identification of the Ph chromosome. Different groups have reported FISH sensitivity of 98% with false positive results of 2.3 to 2.8% [42]. However, the observed false positivity of FISH is usually clinically non-significant, because it is always backed by cytogenetic metaphase and RT qPCR analysis of PB or BM cells.

The validation of the results is a very important part of the screening process due to the risk of false positivity associated with the PCR-based methods. The majority of the methods for PFG screening are RNA-based techniques consisting of analysis of cDNA that was *in vitro* reversely transcribed from the particular mRNA molecules. Due to the occurrence of the *trans*-splicing phenomenon, although at extremely low frequency, these methods may identify preleukemic fusion transcripts even in the absence of corresponding chromosomal translocation, thus producing false positivity.

Therefore, the DNA-based methods, such as FISH or flow FISH would be preferable, although their sensitivity is too low when we assume relatively very low amount of positive cells/signal present in analyzed samples. The trade-off is the analysis of fusion transcripts. They can be verified by sequencing of the PCR products to prove formation of a correct, i.e. *in frame* fusion between the two hybrid genes. In addition, the data might be further validated by repetitive screening performed by either the same or different group (e.g. reference or certified laboratory). Table 1 summarizes validation of *BCR-ABL* incidences which was performed in limited number of studies, including Biernaux *et al* (1995) and two reports from our laboratory [26, 28]. Direct sequencing of *BCR-ABL* p210 PCR-products by Biernaux and colleagues revealed the presence of correct sequence junction between BCR exon3 and ABL exon2 (junction b3a2) in 100% p210+ (23/23) samples [36]. Similarly, our group reported high percentage of validation of *BCR-ABL* p190 by sequencing, namely 90.1% (20/22) [28]. In addition, we reported a repeated RT qPCR screening of selected *BCR-ABL* p190+ samples performed either in a reference laboratory located outside with 26.7% (4/15) concordance [26] or in our laboratory achieving similar validation rate, namely 30% (27/90) [28]. These data suggest that approximately 1/3 of p190+ samples have been confirmed by repeating the screening using the same RT qPCR method. This relatively low validation rate could be explained by an

extremely low copy number of the p190 PFG in the analyzed samples, which was close to the threshold of the sensitivity of the screening method (1 – 3 x 10^{-5} for RT qPCR in our laboratory), therefore limiting determination of the exact PFG values. It is obvious that both these validation methods cannot confirm the presence of corresponding chromosomal translocation as it FISH method allows. However, FISH technique cannot be applied for PFG screening in UCB mononuclear cells (MNC) due to its insufficient sensitivity.

The available data on the incidence of *BCR-ABL* are summarized in Table 1. In the case of *BCR-ABL* p210, these data suffer from relatively low statistical power, with an average number of screened samples less than 50. After exclusion of the Song's report showing several-fold higher frequencies of PFG than all other studies, we take into consideration results of five reports. In children's PB, the overall p210 incidence may be estimated in the range between 4.5% up to 9.1%, ~ 7% [30, 36]. In adult's PB, the incidence may be enhanced from 6 % up to 30%, ~ 18% [30, 36, 37, 39]. The frequency of *BCR-ABL* p210 in UCB cannot be estimated due to insufficient data, however, the data of Biernaux and colleagues reporting none positives among 22 samples [36] may suggest a significantly lower p210 incidence in cord blood than in children, thus supporting the upward tendency of p210 incidence with age. Based on the results published in two recent studies by our group, the incidence of *BCR-ABL* p190 may be estimated to about 5 – 6% in UCB [26, 28]. These data are supported by (i) sufficient statistical power of the two studies, counting 200 and 500 screened samples, respectively, (ii) using a screening method with extremely low risk of cross-contamination, and (iii) applying several levels of data validation. On the other hand, the data on the incidence of *BCR-ABL* p190 in both children and adults are insufficient, and obviously new studies are required to validate the aforementioned estimates.

TEL-AML1 fusion gene. The incidence of *TEL-AML1* has repeatedly been investigated in UCB of healthy individuals (Table 2). In 2002, the study of Mori *et al.* has reported that the incidence of *TEL-AML1* fusion transcript in UCB is 1.05% [2]. This study was the first one to detect PFG in healthy individuals using RT qPCR. This method reached a sensitivity of 10^{-4} and confirmed all positive results obtained by the initial nested RT PCR (6/567) at the same sensitivity [2]. The reported results seem to be very reliable due to high statistical power of the study (567 probands) although a risk of cross-contamination in the first nested RT PCR cannot be excluded. Moreover, this group used a flow cell sorter and immunomagnetic beads to sort subpopulations of T lymphocytes (CD3+), B cell lineage (CD19+), monocytes (CD14+), and rare HSC/PC (CD34+) populations from 4 positive UCB samples, followed by nested RT PCR (in 1 sample) or FISH analyses (in 3 samples). Nested RT PCR revealed positivity in sorted CD19+ and CD34+ subpopulations. These cell populations represent respectively 80% and 1.5% of non-sorted UCB MNC, while both membrane markers, CD19+ and CD34+, usually characterize blasts from children with *TEL-AML1* B-precursor ALL.

Table 2. Incidence of *TEL-AML1* in healthy subjects

Studies	Source	Methods	Sensitivity	*TEL-AML1* positive/ examined subjects (incidence, %)	Comments (Age)	Analyzed sample
Eguchi-Ishimae (2001)	UCB PB	Nested PCR Nested PCR Sequencing	10^{-5} 10^{-5}	1/67 (1.5%) 11/99 (11%) 2/48 (4%) 13/13 (100%)	< 20 years > 20 years	MNC Total RNA
Mori (2002)	UCB	RT qPCR Nested PCR Sequencing Sorting + FISH	10^{-4} or above 10^{-4}	6/567 (1%) 6/567 (1%) 6/6 (100%) 2/3 (66%) In total ~1%		1×10^{6} MNC $4\mu g$ RNA to cDNA $1\mu l$ cDNA (1/40 cDNA) per RT qPCR reaction $2\mu l$ cDNA/1 round, $1\mu l$ cDNA/2 round RT PCR
Olsen (2006)	PB	RT qPCR 1 RT qPCR 2 Nested PCR Dot blot Sequencing	10^{-4}-10^{-5} 10^{-4}-10^{-5} 10^{-4}-10^{-5}	13/2005 (0.6%) tube A 0/13 (0%) tube B 0/5 (0%) tube B 10/13 (76%) tube A 3/9 (30%) tube A In total < 0.5%	Adults	$\geq 1.6 \times 10^{6}$ MNC $12\mu l$ (24%) mRNA to cDNA $10\mu l$ cDNA (12% mRNA) per RT qPCR reaction $5\mu l$ cDNA/1 round, $1\mu l$ cDNA/2 round RT PCR
Lausten-Thomsen (2008)	BM from embryos tissues	RT qPCR Sorting	10^{-4} to 10^{-5}	0/27 (0%)	43-66d	MNC – within 2-4h
Lausten-Thomsen (2010)	UCB (prematurely born children)	RT qPCR1 Dot blot RT qPCR2	10^{-4}-10^{-5} 10^{-4}-10^{-5}	1/256 (0.4%) 0/1 (0%) 0/1 (0%)		Median 7.8×10^{6} MNC $12\mu l$ (24%) mRNA to cDNA $10\mu l$ cDNA (12% mRNA) per RT qPCR
Zuna (2011)	UCB Aborted fetuses (liver and spleen)	Nested PCR or RT qPCR FISH	NA NA NA NA	5/253 (2%) 1/5 (25%) 1/12 (8%)		NA
Lausten Thomsen (2011)	UCB	RT qPCR 1. RT qPCR 2. Dot blot Sorting+RT qPCR	10^{-4}-10^{-5} 10^{-4}-10^{-5}	14/1417 (0.9%) 0/14 (0%)/ 9/14(64%)/0.6% 0/14(0%)	Tube A Tube B Tube A Cells in N_2	$\geq 2.5 \times 10^{6}$ MNC $18\mu l$ (36%) mRNA to cDNA $10\mu l$ cDNA (12% mRNA) per RT qPCR reaction
Olsen (2012)	UCB	RT qPCR 1 RT qPCR 2 Dot blot Sequencing	10^{-4}-10^{-5} 10^{-4}-10^{-5}	3/1258 (0.24%) 0/3 (0%) 3/3 (100%) 2/3 (66%)	Tube A Tube B Tube A Tube A	$\geq 1.6 \times 10^{6}$ MNC $12\mu l$ (24%) mRNA to cDNA $10\mu l$ cDNA (12% mRNA) per RT qPCR
Barbany (2013)	PB from GC	RT qPCR	10^{-3}-10^{-4}	0/30(0%)		RNA from 6×10^{5} MNC
Skorvaga (2014)	UCB	RT qPCR 1 RT qPCR2 Nested PCR Multiplex PCR	$1\text{-}3 \times 10^{-5}$ $1\text{-}3 \times 10^{-5}$ $1\text{-}3 \times 10^{-5}$ $0.2\text{–}1 \times 10^{-3}$	P190 32/200 (16%) P190 4/15 (26.6%) In total 4% P190 0/135 (0%)		10^{7} MNC $1\mu g$ total RNA to RT $2\mu l$ cDNA *per* RT qPCR reaction
Ornelles (2015)	UCB	Nested PCR Sequencing	NA	5/210 (2.4%) 5/5 (100%)		10^{6} lymphocytes 200ng RNA to RT $5\mu l$ cDNA/1 round, $3\mu l$ cDNA/2 round RT PCR
Kosik (2015)	UCB	RT qPCR 1 RT qPCR 2 Sequencing	$1\text{-}3 \times 10^{-5}$ $1\text{-}3 \times 10^{-5}$	45/500 (9%) 27/90 (30%) 20/22 (90.9%) In total 2.4%		10^{7} MNC $1\mu g$ total RNA to RT $2\mu l$ cDNA *per* RT qPCR reaction

PB – peripheral blood; MNC – mononuclear cells; RT – reverse transcription; RT PCR – nested PCR; NA – non available; A – tube A; B – tube B

Using cell sorting, this study reached much higher sensitivity. FISH analysis of CD19^{+} cells for *TEL-AML1* also confirmed positivity in 2 out of 3 positive MNC samples at a similar level of 0.25-0.33%. The results by Mori *et al.* [2] are comparable to the data of Eguchi-Ishimae *et al.* [43] who have determined incidence of *TEL-AML1* equal to 1.5% (1/67) using nested RT PCR with a sensitivity of 10^{-5}, which was one order of magnitude higher than that used in study by Mori *et al.* [2]. Although the positive product has been directly sequenced by Eguchi-Ishimae *et al.*, the weaknesses of this study design was in low number of UCB samples (67) and increased risk of contamination caused by two-step nested RT PCR. In line with this study, the Czech group of Trka has reported very similar, 2%, incidence of *TEL-AML1* derived from 253 UCB samples [27]. These UCB have been analyzed by either nested RT PCR or RT qPCR, although sensitivity of both methods has not been provided and the methods have not been described. One positive sample was confirmed by FISH, where 3000 nuclei were screened and 3 of them (0.1%) contained visible *TEL-AML1* signal. Although the study did not find any *TEL-AML1* positive signal by analyzing 5000 negative control cells this number is lower than the false *BCR-ABL* positive rate of 2.3% determined by FISH in cells from healthy donor [44]. Following these results, Skorvaga *et al.*, Ornelles *et al.*, and Kosik *et al.* reported slightly higher frequencies of *TEL-AML1* in comparison to the Mori´s study, corresponding to 6.25%, 2.4%, and 5%, respectively [26, 28, 45]. The first study from our laboratory by Skorvaga *et al.* has screened 200 UCB by RT qPCR with a detection limit of 1-3 cells positive among 100,000 cells (10^{-5}). The data were validated in the reference laboratory by screening 20 positive UCB samples resulting in 25% (5/20) validation rate. The second study from our laboratory by Kosik *et al.* was the extension of the first study with the aim to get higher statistical power and refine validation rate. In this study, 500 UCB have been screened and 90 samples were validated by RT qPCR. Additionally, validation by sequencing 20/22 (90.01%) has been included. The study by Ornelles *et al.*, which employed nested RT PCR assay, suggested that 2.4% (5/210) of newborne's UCB contained the *TEL-AML1* translocation. This study used statistical power of 210 samples and validation by sequencing 5/5 (100%). However, sensitivity of nested RT PCR was not reported.

Contrary to all aforementioned reports, ~100-fold lower frequencies of *TEL-AML1* fusion transcripts have been observed in three studies from the same Danish group. In the first of these studies, UCB was collected from prematurely born children [46]. Initial screening by RT PCR detected only 1 out of 256 samples as *TEL-AML1* positive (0.4%) at Ct value 44.4 below the study detection level 10^{-5}. The positive product was not confirmed by dot blot hybridization and secondary RT qPCR. The second Danish study initially found ~1% (14/1417) incidence of *TEL-AML1* in fresh UCB cells processed within 24 hours [3]. After using dot blot hybridization, from fourteen originally positive samples, only nine were confirmed as positive and thus established the incidence

being 0.6%. Re-screening of all positive samples by RT qPCR analysis using second tubes of the same mRNA stored at -80°C did not confirm any fusion transcripts. Similarly, flow cytometric sorting of a B cell lineage (CD19^{+}), T cell lineage (CD8^{+}) and remaining CD19^{-}/CD8^{-} subpopulations from cryopreserved MNC, representing in average 0.99, 0.613, 6.755 x 10^6 cells, respectively, revealed no positive findings. Finally, the incidence of *TEL-AML1* was estimated to be less than 0.01%. The third study from this group obtained similar results [25]. This study reported the presence of *TEL-AML1* transcripts in 3 UCB out of 1258 (0.24%) samples. In contrast to the previous Danish study, only 9 out of 1258 (0.7%) samples were processed until 24 hours and most of them much later: 24-48 hours (680), 49-72 h (288), 73-96 h (316) and > 96 h (15). A criterion for selection of non-degraded mRNA, based on the comparison of RT qPCR amplification efficiency between *TEL-AML1* and ABL control gene, was ABL Ct ≤ 24.8. This value would allow detection of *TEL-AML1* at the levels of 10^{-4} and the Ct value of 38. The three samples tested positive by RT qPCR were processed between 24-48 h, and thus in 0.8% of 378 UCB processed within 48 h *TEL-AML1* fusion gene were determined. Dot blot confirmed positivity in all 3 samples (100%), whereas sequencing only in 2 of them (66%), with no samples being positive in a repeating RT qPCR analysis 0/9 of mRNA stored at - 80 °C. The strength of the Danish studies included: (i) high statistical power of 256, 1417 and 1258 samples, (ii) testing mRNA instead of RNA (iii) RT qPCR analysis in sorted subpopulations (in one study) (iv) using high amount of cDNA for RT qPCR ((10μl cDNA (16%), 7 times as much mRNA-derived cDNA as Mori *et al.* in first round PCR screening), (v) higher sensitivity of PCR detection (10^{-5}) than that by Mori *et al.* (10^{-4}) [2] but similar to the studies by Skorvaga and Kosik *et al.* (10^{-5}) [26, 28]. On the other hand, limitation of these studies was validation by post PCR dot blot hybridization without providing sensitivity for *TEL-AML1* detection fusion.

Very few studies assessed *TEL-AML1* in PB of: (i) neonates [47], (ii) children [43], and (iii) adults [43, 48]. Eguchi-Ishimae *et al.* have detected that 11% (11/98) and 4% (2/18) of PB from children and adults over 20 years, respectively, were *TEL-AML1* positive [43]. Therefore, no correlation with age has been found [43]. Due to the low number of tested samples, the incidence of *TEL-AML1* fusion gene cannot be reliably estimated from these data. Olsen *et al.* demonstrated significantly lower incidence of *TEL-AML1* transcripts in 2005 adult's PB (< 0.5%), with a note that their second round of RT qPCR (0/13) and nested RT PCR (0/5) with the same sensitivity, from 10^{-4} to 10^{-5}, failed to confirm the results obtained in the first round of single RT qPCR (13/2005, 0.65%); dot blot (10/13, 76%) and sequencing (3/9, 33%) significantly differed in their validation efficiency [48]. This study had the highest statistical power among all *TEL-AML1* studies. However, limitation of this study is in using a single reaction for the initial RT qPCR screening, instead of commonly used triplicates. No *TEL-AML1* transcript was detected by Barbany *et al.* in RNA

isolated from the PB dropped on GC [47]. However, a number of subjects (30) enrolled in this study was too low. In addition, the RT qPCR sensitivity was relatively low, from 10^{-3} to 10^{-4}, and storage conditions could lead to RNA degradation limiting the reliability of this study.

TEL-AML1 fusion transcript was detected in the spleen of one of twelve amortized fetuses (8%) [27]. No one of human embryos livers (0/27) which contained in average 69.6% CD34+ cells were tested positive for *TEL-AML1* [49]. However, these studies involved insufficient number of tissue samples to make any conclusion about incidence.

To conclude, while three studies from the same Danish group inclined towards the incidence of PFG identical to the incidence of *TEL-AML1*+ ALL in children, four independent studies from different laboratories [2, 27, 43, 45] and two our studies [26, 28] reported much higher incidence of *TEL-AML1* (~100-fold) than incidence of overt childhood *TEL-AML1*+ leukemia (Table 2). Except for publications by Mori *et al.* [2] and Lausten-Thomsen *et al.* [3] a common limitation of these findings is that the relevance of the cell type harboring the fusion genes to the origination of leukemia was not elaborated.

MLL-AF4 fusion gene. Up to now, only three original articles, one study presented at the European Cancer Congress in 2015 and two letters to the editor have presented data on *MLL-AF4* fusion transcripts in healthy individuals (Table 3). Letters to the editor provided only limited information regarding results and methods [50, 51]. Two our studies, where

the RT qPCR has been used for the primary screening UCB MNC, followed by validation analysis as is described in the chapter of *TEL-AML1* fusion gene, found *MLL-AF4* transcripts in 0.75% and 0.8% samples [26, 28]. Two other studies which used nested RT PCR with the same sensitivity as we used (10^{-5}) did not detect any *MLL-AF4* in UCB (0/60 and 0/103, respectively) [50, 51]. Of note, these studies analyzed much lower number of subjects as compared to our studies. Song *et al.* observed that *MLL-AF4* occurred with higher frequency 80% (8/10) in PB of newborns [35], while the other age groups showed a lower range, moving from 40% to 69% (52% in children vs adults). The limitations of this study were mentioned in the chapter *BCR-ABL* fusion gene. Besides PB and UCB, other sources such as fetal liver, fetal BM or neonatal BM have been analyzed. The incidence of *MLL-AF4* transcripts was observed by nested RT PCR at high frequency in fetal liver (5/13; 38%), fetal BM (4/16; 25%) and normal infant BM (1/6; 17%) by Uckun and colleagues [38]. Although at relatively low statistical power, this study achieved a notable specificity by using complementary detection and validation methods including: (i) standard cytogenetic technique, (ii) Southern blot analysis of selected samples, namely 7/9 rearrangements resulting in *MLL* genomic disruption, (iii) re-analysis of 15/17 cytogenetically negative samples by nested RT PCR, and (iv) sequence analysis of selected *MLL-AF4*+ samples, including infants (n = 3) or children (n = 15) with ALL, fetal liver (n = 5) and fetal BM (n = 2). However, these data were not confirmed by the study of Kim-Rouille *et al.*, who detected no positive

Table 3. Incidence of *MLL-AF4* gene in healthy subjects

Studies	Source	Methods	Sensitivity	*MLL-AF4* positive/examined subjects (incidence, %)	Comments (Age)	Analyzed sample
Uckun (1998)	Fetal liver	Nested PCR	10^{-4}	5/13 (38%)	Gestational age 15-22 weeks	Total RNA
	Fetal BM	Nested PCR	10^{-4}	4/16 (25%)		
	Neonatal BM	Nested PCR	10^{-4}	1/6 (17%)		
		Standard PCR	10^{-2}	0/35 (0%)		
		Southern blot		0/10 (0%)		
Trka (1999)	UCB	Nested PCR	10^{-4}	0%		Total RNA
Kim-Rouille (1999)	UCB	Nested PCR	10^{-5}-10^{-6}	0/60 (63%)		mRNA
	Fetal liver			0/8 (0%)		
	Fetal BM			5/NA (NA%)		
		Sequencing		0/5(0%)	Unrelated products	
Song (2011)	PB	Nested PCR	10^{-4}	8/10 (80%)	Newborns	8-16x10^6 MNC
				13/25 (52%)	Children < 25 years	0,5g total RNA for RT
				19/36 (52%)	Adults > 25 years	3μl cDNA/1 round, 1μl cDNA/2 round RT PCR
Skorvaga (2014)	UCB	RT qPCR 1	1-3 x 10^{-5}	P190 6/200 (3%)		10^7 MNC
		RT qPCR 2	1-3 x 10^{-5}	P190 4/15 (26.6%)		1μg total RNA to RT
				In total 0.75%		2μl cDNA *per* reaction
		Nested PCR	1-3 x 10^{-5}			
		Multiplex PCR	0.2–1 x 10^{-3}	P190 0/135 (0%)		
Kosik (2015)	UCB	RT qPCR 1	1-3 x 10^{-5}	16/500 (3.2%)		10^7 MNC
		RT qPCR 2	1-3 x 10^{-5}	27/90 (30%)		1μg total RNA to RT
		Sequencing		20/22 (90.9%)		2μl cDNA *per* reaction
				In total 0.8%		

MNC – mononuclear cells; RT – reverse transcription, NA – non available

sample of fetal liver (0/8) despite using nested RT PCR with significantly higher sensitivity (10^{-5}-10^{-6}) [51]. The necessity of applying several complementary validation techniques, especially in clinical practice, can be documented by the case report of a 30-year old *MLL-AF4*$^+$ patient with high risk ALL [52]. Detailed investigation revealed the presence of (i) normal karyotype, 46 XY (cytogenetic analysis), (ii) genomic disruption of *MLL* (Southern blot), (iii) common *MLL-AF4* fusion transcript (RT PCR confirmed by sequencing), and (iv) insertion of 5' *MLL* sequence into the AF4 locus of chromosome 4 (high-resolution fiber FISH). The patient presented with a high blast cell count, a pre-B phenotype and central nervous system involvement which are classical features of t(4;11)-ALL. However, the karyotype was normal, since the insertion was submicroscopic. A similar insertion mechanism might also occur in *BCR-ABL* fusions as it has been suggested for the Philadelphia negative *BCR-ABL* fusions in CML [53, 54]. These cases indicate that even cytogenetically undetectable chromosomal translocations positive for a functional fusion transcript may result into leukemogenesis, thus contributing to the complexity of the screening procedures.

As mentioned in the *BCR-ABL* fusion gene section, the results and conclusion stemming from the Uckun's report [38] are limited due to small number of screened samples and use of a method with an increased risk of false positivity, although authors stated the use of strict precautions to prevent cross-contamination. Similarly, the sample size of the study by Kim-Rouille *et al*. [51] counting only 60 UCB and 8 fetal

liver samples was too low. The same is valid for the study by Song *et al*. with 71 samples. From this point of view, only the study of Trka *et al*. [50] and two our studies [26, 28] provide sample size, namely 103, 200, and 500, respectively, ensuring appropriate statistical power to estimate PFG incidences that can be compared and from which a reliable conclusion may be drawn. While Trka *et al*. did not find any *MLL-AF4* positivity (0/103) with 1×10^{-4} sensitivity of nested RT PCR, we detected this fusion gene at the incidence of 0.8 % using RT qPCR with sensitivity of $1 – 3 \times 10^{-5}$. Based on higher statistical power of our studies, we conclude that the incidence of *MLL-AF4* in the UCB may be estimated as 0.8 %.

AML1-ETO, PML-RARA, CBFB-MYH11 fusion genes. In few studies, relatively rare PFG characteristic for both ALL and AML were investigated in UCB, PB, and BM of healthy subjects (Table 4). The *AML1-ETO* incidence was determined in UCB as 0.2% (1/496) and 40% (63/156) by the study of Mori *et al*. [2] and the report of Basecke *et al*. [55], respectively. Out from 63 positive samples, 6 samples were positively re-analyzed (6/6, 100%) in the study by Basecke *et al*, however, without indicating the sensitivity of the RT qPCR. The difference in results could be explained by (i) higher sensitivity of nested RT PCR (10^{-6}) used by Basecke *et al*. in comparison to by Mori *et al*. (10^{-4}), (ii) cross-contamination, (iii) low quality RNA extracted from UCB as the authors assume in conclusion. However, no data describing amount of cells used for RNA extraction, quality of RNA and cDNA were reported. Additionally, this study estimated the incidence of

Table 4: Incidence of other PFG in healthy subjects

Studies	PFG	Source	Methods	Sensitivity	PFG positive/examined subjects (incidence, %)	Comments (Age)	Analyzed sample,
Mori (2002)	*AML1-ETO*	UCB	RT qPCR	10^{-5}	1/496 (0.2%)		1×10^6 MNC
			Nested PCR	10^{-5}	1/496 (0.2%)		4μg RNA to cDNA
			Sequencing		1/1		1μl cDNA (1/40 cDNA)
							per RT qPCR reaction
							2μl cDNA/1 round, 1μl
							cDNA/2 round RT PCR
Basecke (2002)	*AML1-ETO*	UCB	Nested PCR	10^{-6}	63/156 (40%)		NA
			RT qPCR	NA	6/6(100%)		
		BM			4/18 (22%)	Adults: 22-76 years	
Song (2011)	*AML1-ETO*	PB	Nested PCR	10^{-4}	1/10 (10%)	Newborns	8-16x10^6 MNC
					5/25 (20%)	Children < 25 years	0,5g total RNA for RT
					7/36(19.5%)	Adults > 25 years	3μl cDNA/1 round, 1μl
	PML-RARA	PB			7/10 (70%)	Newborns	cDNA/2 round RT PCR
					10/26 (38%)	Children < 25 years	
					20/38 (52%)	Adults > 25 years	
	CBFB-MYH11				1/10 (10%)	Newborns	
					0/26 (0%)	Children < 25 years	
					1/32 (3.1%)	Adults > 25 years	
	MLL-PTD	UCB	Nested PCR	10^{-3}	40/53 (75%)		
		PB	Nested PCR	10^{-3}	11/14 (79%)	Newborns	
					32/51 (62%)	Children < 25 years	
					39/56 (69%)	Adults > 25 years	

PB – peripheral blood; MNC – mononuclear cells; UCB – umbilical cord blood; RT – reverse transcription; RT PCR – nested PCR; NA – non available

AML1-ETO in BM of healthy adults at 22% (4/18). The study of Song *et al* determined the *AML1-ETO* incidence in PB at 18% (13/71) and the sensitivity of nested RT PCR at 10^{-4} [35]. The highest incidence was recorded in probands within the age group of 26-40 years (5/10, 50%), however, no significant age correlation was observed. The frequency of *PML-RARA* and *CBFB-MYH11* has not been investigated in UCB so far. These PFG have been analyzed only in PB where *PML-RARA* and *CBFB-MYH11* were detected in 50% (37/74) and 3% (2/68) of samples, respectively [35] (Table 4).

Due to considerable limitations such as lack of appropriate validation steps, increased risk of cross-contamination of the applied method and small number of samples analyzed in the study of Song *et al* it is premature to draw any conclusion. Moreover, substantial differences in the incidence of AML1-ETO between studies by Mori *et al*. and Basecke *et al*. do not allow to reliably estimate the incidence of this PFG in UCB.

Discussion

A number of studies have demonstrated the occurrence of PFG in the blood of seemingly healthy individuals (table 1, table 2, table 3 and table 4). In this review, we have compared and summarized the results of reports studying the incidence of PFG typically associated with acute/chronic leukemia in UCB, BM, and PB of healthy subjects. Moreover, we have reviewed a few case reports of asymptomatic or leukemic patients to demonstrate the complexity of diagnostic and screening process. We have focused on the methodological aspect of the screening approaches with the accent on the statistical power of data, the sensitivity and specificity of the screening techniques, and the validation of the data. By applying all these criteria, we were able to make estimations of PFG incidence among healthy subjects as follows: (i) *BCR-ABL* p210 in peripheral blood at ~ 7% in children, and ~ 18% in adults, supporting an upward trend of p210 incidence with age, which may be accounted for age dependent genetic instability (ii) *BCR-ABL* p190 in UCB at ~ 5 – 6%, (iii) *TEL-AML1* in UCB at ~ 1 – 2 %, and (iv) *MLL-AF4* in UCB at ~ 0.8%. In general, there is a notable variation of the PFG incidence in healthy subjects regardless of sample source (UCB, BM and PB) and age group (newborns, children, and adults) studied. Several factors may contribute to the observed variation, including: (i) relatively low statistical power and low number of studies, (ii) inconsistent methodology in terms of diverse sensitivity, specificity and susceptibility to contamination of applied techniques, (iii) insufficient or even absent validation of the results in some reports, and (iv) ethnic, geographical or environmental factors. In particular, the differences in sample processing and methodology may have a substantial influence on the final results even when the same PCR-based method used in different laboratories. Here are some of the major methodological issues: (i) cell type used, (ii) analysis of total RNA *versus* mRNA, (iii) different amount of cells used

for RNA isolation ($0.8x10^6$-$1x10^8$) and (iv) various quantity of RNA used for reverse transcription (0.5-10μg). Some reports did not clearly specify the amount of RNA used in RT reaction, e.g. showing only its volume without indicating the concentration of RNA. There was a marked variability in the sensitivity assessment of the applied method among different groups, for example different molecules or cells were tested and diluted, such as DNA in DNA, RNA in RNA, negative cell line in positive cell line, or cells from leukemia patients in cells from healthy donors. After taking into consideration all above mentioned factors and differences in the reviewed reports, it is not surprising that the sensitivity of nested RT PCR and RT qPCR has been reported in the range of 10^{-4} to 10^{-8} and 10^{-4} to 10^{-5}, respectively.

Overall, the data available in scientific literature indicate that the PFG frequencies in healthy subjects vastly exceed the incidence of leukemia in human population. In general, the data on the incidence of *TEL-AML1* in UCB support Model A which assumes that the initiating genetic event, i.e. t(12;21) chromosomal translocation resulting into *TEL-AML1* fusion occurs at relatively high proportion (~1%) of newborns [56]. Taking into consideration the cumulative incidence of *TEL-AML1*+ ALL in children (1:10,000, i.e. 0.01%), it predicts that ~1 of 100 newborns harboring detectable *TEL-AML1* transcripts are destined to develop ALL [56]. Even lower proportions between PFG in cord blood and acute pediatric leukemia incidences have been reported for *BCR-ABL* and *MLL-AF4*.

The relevance of studies on PFG screening from the point of risk for leukemia represents the fundamental question. There is a compelling evidence, including retrospective analysis of GC, that several PFG associated with specific chromosomal translocations and acute pediatric leukemia, i.e. *MLL-AF4*, *TEL-AML1*, and AML1-ETO often originate prenatally *in utero* during embryonic/fetal development [57, 58, 59]. However, most of the supporting studies on GC reported the presence of specific chromosomal translocations in limited proportion of studied cases. For example, in one of the first such study the authors were able to detect the t(12;21) chromosomal translocation resulting into TEL-AML1 in 9/12 Guthrie cards from children with *TEL-AML1*+ B-ALL [60]. Other reports show the results, e.g. 3/3 for *MLL-AF4* without positivity in all tested segments [24], or 5/10 for t(8;21)/AML1-ETO in GC from AML children [61]. These negative neonatal GC/segments could either indicate a postnatal origin of the translocation or more likely its prenatal origin that could not be detected due to (i) insufficient number of positive cells present in the blood spot examined, (ii) degradation of DNA in the sample, or (iii) presence of PCR inhibitors in the tested sample. Therefore, due to these technical drawbacks, blood-spot screening may underestimate the frequency of cases that are originated before birth.

Due to an *in utero* origin of many PFG, the screening of UCB for the presence of chromosomal translocations commonly associated with leukemia in children as well as in adults

and giving rise to leukemia-initiating cells (LIC) or cell-of-origin may be the most valuable in terms of risk prediction/estimation for leukemia and donor-cell derived leukemia (DCL) before allogeneic cord blood transplantation .

With two exceptions, the majority of studies on PFG incidence in UCB so far have been performed on UCB MNC regardless the leukemogenic potential of various cell populations. Thus, the research must be focused on identification of LIC. Although LIC may not be identified for all types of leukemia, these cells must possess the essential stem cell characteristics: (i) self-renewal and (ii) indefinite proliferative potential, which can give rise to leukemic stem cells (LSC) that initiate and maintain the disease. The data have suggested that hematopoietic stem cells (HSC) serve as the cancer-initiating cells for LSC [62, 63]. It has been demonstrated that all clonogenic capacity resided in Lin$^-$ CD34$^+$ CD38$^-$ AML cells, in contrast to Lin$^-$ CD34$^+$ CD38$^+$ or Lin$^+$ AML cells that failed to induce leukemia [64, 65]. In addition, it has been shown that, the *BCR-ABL*1 fusion protein can be detected in several hematopoietic lineages of CML patients, suggesting that cell-of-origin can be also an early multipotent progenitor (MPP), i.e. an HSC with multilineage differentiation potential [66, 67, 68]. Many lines of evidence support the concept that other more differentiated cells can give rise to LSC after re-acquisition of self-renewal (progenitor cell-of-origin) [69, 70]. Moreover, the recent demonstration of some AML LSC expressing even low amounts of lineage markers raised question whether more differentiate hematopoietic cells may serve as cell-of-origin for LSC as well [71]. In case of ALL, it has been demonstrated that expression of *TEL-AML1* fusion transcript was detected in a rare population of CD34$^+$/CD38$^{-/low}$/CD19$^+$ cells (0.002%) [72]. In addition, Singer and colleagues showed that B lineage progenitors, including the pro-B cell Lin$^-$CD19$^+$/CD45R$^+$/CD43$^+$/CD93$^+$/IgM$^-$ efficiently initiate *BCR-ABL* B-ALL after transplanting them into sublethally irradiated *Rag1*$^{-/-}$ mice [73]. Altogether, these results are encouraging in efforts to identify cell-of-origin for LSC in both ALL and AML.

Obviously, screening MNC from UCB and similarly, BM and PB, cannot give the answer to the question how the identified fusion genes will increase the risk for leukemia in their carriers during their development. Depending on how many samples tested positive for a particular PFG actually contain the corresponding chromosomal translocation in the HSC/PC, i.e. in cells with leukemogenic potential, the resulting risk for leukemia can be in the range from 0 to 100%. Again, this is a very broad interval and the screening performed in cell populations with self-renewal potential may greatly reduce this range. An intriguing question may be how many children carrying a long-term HSC (a very primitive, extremely rare stem cell in G$_0$ phase) stricken by 1st hit will get leukemia *versus* those children in which the initial translocation arose in short-term progenitor cell. We assume that further research on identification of cell-of-origin for ALL, AML and CML will continue and hopefully will allow us to define the cell populations within UCB that can give rise to LSC. We think that it

is necessary to introduce new screening approaches which would allow to target leukemogenic cells, i.e. hematopoietic stem/progenitor cells and to exclude more mature and differentiated cells which are not capable of self-renewal.

The research effort should be directed towards characterization and definition of the target cell populations where the preleukemic lesions initially arise. In this way, the specificity of the screening will be much higher and RNA-based methods as for example qPCR could be substituted with DNA-based methods such as flow-FISH, thus further increasing the specificity of the screening by reduction of false positives that might be formed by alternative splicing. In the context of acute leukemia, it is generally accepted that the process of malignant transformation, i.e. generation of a neoplastic clone with deregulated growth properties, involves the acquisition of several genetic and epigenetic alterations likely in HSC/PC. These alterations lead to conversion of normal HSC/PC into a LSC which is capable of propagating the leukemic clone. However, these LSC are quite rare and their numbers are different depending on their origin. In RT qPCR which was used for PFG screening of UCB in our laboratory, normally 1µg of total RNA was used for cDNA synthesis which at an average concentration of 1 – 2 pg RNA/cell is equivalent to 500,000 – 1,000,000 MNC. As a standard, 1/10 of cDNA was applied in subsequent RT qPCR which is equivalent to 50,000 – 100,000 MNC. In average, 6 copies of PFG$^+$ *per* positive sample was identified among 100,000 MNC, corresponding to ~ 1000 CD34$^+$ cells among which ~ 4% may represent more primitive CD34$^+$ CD38$^-$ cell population, inhibited in G$_0$ phase [74]. It means that if we find e.g. ~ 6 copies of TEL-AML1$^+$ PFG *per* 100,000 UCB MNC, using CD34$^+$ CD38$^-$ cell population would require to identify ~ 6 positive signals among 120 cells, therefore, flow-FISH technique could be used for an efficient and unambiguous PFG screening with a possibility to estimate the risk of leukemia.

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