Novel molecular genetic insights in paediatric B-cell precursor acute lymphoblastic leukaemia

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SUMMARY

Paediatric B-cell precursor acute lymphoblastic leukaemia arises from recurrent genetic lesions that block precursor B-cell differentiation and drive aberrant proliferation and cell survival. Risk-adapted intensive chemotherapy has been a major breakthrough in reaching the current survival rates of >90% for this ALL subtype. Recent developments in genome-wide genetic analysis have provided a wide range of chromosomal and genomic abnormalities characterising B-cell precursor acute lymphoblastic leukaemia, several of which are associated with patient outcome. This article summarises the results of several studies performed during the PhD thesis of Dr Farzaneh Ghazavi. This research project has led to the identification of a novel molecular lesion predicting poor outcome, a novel targetable pathway in a subgroup of B-cell precursor acute lymphoblastic leukaemia patients and resulted in the identification of an ETV6/RUNX1-specific long non-coding RNA signature providing novel biological insights into ETV6/RUNX1-mediated leukemogenesis.

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INTRODUCTION

B-cell precursor acute lymphoblastic leukaemia (BCP-ALL) is the most common paediatric malignancy and the cure rate of this disease has increased enormously over the last forty years to a current five-year survival of over 90%, attributed to improved chemotherapeutic treatment schedules and risk-stratification.[1] Nevertheless, some patients still experience treatment failure after initial therapy and therefore, identification of novel biomarkers is critical to distinguish these patients at diagnosis, adapt their treatment schedules and to develop novel strategies using new therapeutic targets.

Recent developments in whole genome techniques (DNA, RNA, and protein) provided detailed information concerning the molecular alterations important for tumour emergence, maintenance and progression. Several groups have demonstrated the power of high-resolution, genome-wide approaches to identify new molecular lesions in leukaemia. As such, deletion, amplification, point mutation and structural rearrangement in genes encoding principal regulators of B lymphocyte development and differentiation were identified in 40% of B-progenitor ALL cases using single nucleotide polymorphism arrays.[2-5]

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This paper discusses the results obtained through studying the genome, long non-coding repertoire and proteome during the PhD thesis of Dr Farzaneh Ghazavi and provides future perspectives.

IDENTIFICATION OF CD200/BTLA DELETIONS IN PAEDIATRIC BCP-ALL TREATED ACCORDING TO THE EORTC-CLG 58951 PROTOCOL

The advent of high-resolution, microarray-based techniques such as array-based comparative genomic hybridisation (CGH) has enabled the identification of multiple novel DNA copy number alterations targeting key cellular pathways in ALL. Some of the identified genetic lesions are clinically relevant and can be integrated into the risk classification of the ALL patients, as recently reviewed by Ghazavi *et al.*[2-5] Nevertheless; novel prognostic markers need to be discovered, as illustrated by the observation that a considerable number of relapses remain molecularly unexplained.

To identify new genetic lesions with prognostic relevance in BCP-ALL, we performed copy number profiling on leukemic blasts of BCP-ALL patients treated with the EORTC-CLG 58951 protocol. This analysis revealed a recurrent deletion of a genomic region (~164kb) at chr3q13.2, encompassing the lymphoid signalling molecules *BTLA (CD272)* and *CD200* genes, in 10% (7 out of 70) of the initial group and 4.8% (56 out of 1,154) of an extended cohort of BCP-ALL patients.[6] Mapping the exact breakpoint of *CD200/BTLA* deletions in primary leukaemia genomes demonstrated that the deletions mostly affected the whole genomic sequence of both *CD200* and *BTLA* genes and that the deletions were likely mediated by abnormal RAG activity, as evidenced by the RAG recognition sequence motif adjacent to the genomic breakpoints. In line with recent reports that identified aberrant RAG recombinase activity as one of the main mechanisms that drive the generation of cooperative genomic lesions in *ETV6/RUNX1*-positive BCP-ALL, we found that *CD200/BTLA* deletions were strongly associated with t(12;21) positive leukaemia ($p<0.0001$), suggesting a genetic interaction between loss of *CD200/BTLA* and expression of ETV6/RUNX1 during leukemic transformation.[7] The presence of *CD200/BTLA* deletions did not affect the favourable prognosis of *ETV6/RUNX1*-positive BCP-ALLs. However, and most notably, *CD200/BTLA* deletions were associated with inferior event-free survival, both in the complete patient population (HR 2.02; 95% CI 1.23-3.32; $p=0.005$) as well as in the intermediate genetic risk group (HR 4; 99% CI 1.34-11.93; $p<0.001$). In addition, multivariate analysis confirmed the independent prognostic value of *CD200/BTLA* deletion in the whole cohort of BCP-ALLs. Altogether, these data suggest the added value of CD200/BTLA status in the risk stratification algorithm of BCP-ALL patients.

RPPA-BASED PROTEIN PROFILING IN ETV6/RUNX1-POSITIVE ACUTE LYMPHOBLASTIC LEUKAEMIA PATIENTS WITH LOW CD200 EXPRESSION

Identification of altered signalling pathways is of utmost importance in cancer therapy since many of these pathways are targetable and thus a potential bull's eye for increasing treatment efficiency.[8-10] We focused on the further elucidation of the signalling pathways affected by the recurrent deletion of *CD200* and *BTLA*, which was previously identified.[6] Importantly, both *CD200* and *BTLA* genes, affected by the deletion, encode lymphoid signalling molecules and have been shown to inhibit activation of B-cell receptor (BCR) and MAPK/PI3K signalling, respectively, which are both essential signalling pathways for B-cell proliferation and maturation.[11,12] In addition, activation of BTLA upon interaction with its ligand HVEM, attenuates B-cell proliferation as a result of reduced activation of signalling molecules downstream of BCR. Moreover, crosstalk between CD200 and its receptor CD200R has been shown to mediate the inhibition of Ras/MAPK signalling pathway through recruitment of SHIP and/or RasGAP and subsequently deactivation of signalling molecules such as ERK, JNK, and p38 MAPK.[12]

To further explore the functional consequences of reduced CD200 expression on the activity of downstream signalling pathways, reverse phase protein microarray (RPPA) analyses was applied (Ghazavi *et al.*, in preparation). Using this technique we determined the expression and phosphorylation level of 87 signalling molecules in 72 BCP-ALL patients' specimens. Interestingly, differential expression analysis of proteins/phosphoproteins between patients with low and high expression of CD200 revealed significant upregulation and hyperphosphorylation of proteins involved in PI3K/AKT/mTOR signalling pathway in patients with low CD200 expression. Notably, this pathway is an essential regulator of many cellular processes such as protein synthesis, proliferation and apoptosis and its constitutive activation in many forms of malignancies attests its pathogenetic importance.[13,14] Finally, we evaluated the potential of PI-103, a novel dual inhibitor of PI3Kalpha

KEY MESSAGES FOR CLINICAL PRACTICE

1 **CD200/BTLA deletions are associated with poor prognosis in BCP-ALL.**

2 **PI3K/AKT/mTOR signalling pathway might serve as a targetable pathway for treatment of BCP-ALL patients with low CD200 expression.**

3 **lncRNA expression profiling might reveal new prognostic biomarkers and novel therapeutic targets for the treatment of BCP-ALL patients.**

and mTOR to inhibit growth in CD200 low cells.[15,16] A 72h treatment of REH cells, a cell line carrying a deletion of CD200, showed that PI-103 treatment significantly reduces the phosphorylation levels of AKT, c-RAF and eIF4G and affects survival.

UNIQUE LONG NON-CODING RNA EXPRESSION SIGNATURE IN ETV6/RUNX1-DRIVEN B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKAEMIA

Mammalian genomes comprise ~ 20000 protein-coding genes, only representing <2% of the total genome sequence, whereas more than 90% of the genome is actively transcribed into non-coding RNA (ncRNA). The latter group of RNAs consists of small structural and regulatory RNAs (including rRNAs, tRNAs, snoRNAs and microRNAs) and long non-coding RNAs (lncRNAs, >200 nucleotides).[17,18] More than 10,000 lncRNAs have been annotated so far in the human genome sequence.[19,20] Functional studies, although limited, revealed that lncRNAs regulate many cellular processes including chromatin modification, transcriptional and post-transcriptional regulation, mRNA degradation and translation, and trafficking of molecules across the nuclear membrane.[21-26] Different studies have recently shown that aberrant expression of non-coding RNAs such as long non-coding RNAs (lncRNA) might contribute to the development and progression of leukaemia.[27,28] Identification of disease-associated lncRNAs would not only provide new insights into our understanding of disease mechanisms but might also reveal new prognostic biomarkers or novel therapeutic targets for the treatment of diseases.

Recently, we established an *ETV6/RUNX1*-specific lncRNA signature by performing expression profiling microarray in a set of 64 primary BCP-ALL patients including 25 *ETV6/RUNX1*-positive, seven *TCF3/PBX1*-positive, fifteen hyperdiploid and seventeen normal karyotype BCP-ALLs.[29] The integration of these lncRNA expression data with RNA sequencing results from a panel of thirteen human BCP-ALL leukaemia cell lines, resulted in the identification of sixteen lncRNAs exclusively associated with the presence of the ETV6/RUNX1 fusion protein.

Complemented with shRNA-mediated silencing of endogenous *ETV6/RUNX1* in an *ETV6/RUNX1*-positive BCP-ALL cell line, the list of *ETV6/RUNX1* associated lncRNAs was narrowed down from sixteen to four lncRNAs including *lnc-NKX2-3-1, lnc-TIMM21-5, lnc-ASTN1-1,* and *lnc-RTN4R-1*.

Finally, in vitro knockdown using LNA™ GapmeR technology suggested that *lnc-TIMM21-5* and *lnc-ASTN1-1* are probably not involved in transcriptional regulation but, in contrast, *lnc-NKX2-3-1* and *lnc-RTN4R-1* perturbations resulted in a severe effect on overall transcription.

CONCLUSION

Altogether, the data obtained during this PhD thesis have contributed to the identification of a novel prognostic factor - *CD200/BTLA* deletion - in BCP-ALL, the elucidation of the signalling pathways downstream of these molecules and delivered fundamental proof that lncRNAs play an important role in distinct subtypes of BCP-ALL.

It will be essential to validate the prognostic power of *CD200/BTLA* deletions in an independent cohort. Since our results are clinically relevant, the biotech company MRC Holland (www.mrcholland.com) has decided to include this alteration in their MLPA assays used by many centres for diagnostic work-up of paediatric ALL.

The identification of hyperactive PI3K/AKT/mTOR signalling pathway in BCP-ALLs with low CD200 expression provides a rationale for future experiments using inhibitors of PI3K/mTOR and translation path-

ways in this subgroup of patients. Although our in vitro experiments look very promising, it will be essential to further confirm these findings in vivo using xenograft models of primary patient material.

Finally, the data generated in the last part of the study identified a panel of ETV6/RUNX1-specific lncRNAs that might be implicated in the biology of human BCP-ALL and could serve as novel therapeutic targets for the treatment of this prevalent subtype of human leukaemia. Further functional studies of the identified lncRNAs, including the identification of binding partners, should be performed in order to bring these findings to information impacting on the clinical management of patients.

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