MOLECULAR MECHANISMS INVOLVED IN CHEMORESISTANCE IN PAEDIATRIC ACUTE LYMPHOBLASTIC LEUKAEMIA

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ABSTRACT
Acute lymphoblastic leukaemia (ALL) is the most common paediatric cancer. Despite cure rates approaching 80%, resistance to treatment and disease relapse remain a significant clinical problem. Identification of the genes and biological pathways responsible for chemoresistance is therefore crucial for the design of novel therapeutic approaches aiming to improve patient survival. Mutations in the membrane transporter P-glycoprotein genes, genetic variations in drug-metabolising enzymes and defects in apoptotic pathways are mechanisms of chemoresistance common to a wide spectrum of cancers and also play a role in paediatric ALL. In addition, several recent microarray studies have identified transcriptional profiles specifically associated with chemoresistance and pointed to a number of potentially novel therapeutic targets. These microarray studies have shown that genes discriminating between clinically responsive and resistant leukaemias tend to be involved in cellular processes such as regulation of cell cycle, proliferation, and DNA repair. Here we review the outcomes of these microarray studies and also present our own investigations into apoptotic resistance to DNA double strand breaks (DSBs) in paediatric ALL. We present stratification of paediatric ALL by the profile of DNA damage response following ionising radiation (IR) in vitro. This approach allows classification of ALL tumours at presentation into IR-apoptotic sensitive and IR-apoptotic resistant. Furthermore, apoptotic resistant leukaemias exhibit abnormal response of NFκB pathway following irradiation and inhibition of this pathway can sensitize leukaemic cells to IR-induced DSBs.

Key words: ALL; chemoresistance; DNA damage response; microarray

INTRODUCTION
Leukaemias are the most common cancer in children. Among these, acute lymphoblastic leukaemia (ALL) represents the most frequent subtype, constituting approximately 25% of all paediatric tumours [1]. The disease is characterised by maturation arrest and uncontrolled proliferation of lymphoid progenitors in the bone marrow, and accumulation of malignant lymphoblasts in the bone marrow and peripheral blood. Modern treatment regimens are able to effectively cure ALL in more than 75% of cases [1]. This is accomplished by careful stratification of patients at diagnosis to optimise risk-directed therapy with multiagent chemotherapeutic drug regimens [2, 3]. However, high incidence of the disease renders even the relatively low percentage of treatment resistance considerable clinical burden, estimated to be around 9.3 cases/106 children annually [4, 5]. The vast majority of relapses occur due to failure of therapeutic drugs to completely eradicate the original leukaemic clone and its subsequent re-expansion. Over 70% of relapses happen within 3 years of initial diagnosis [5], and are likely to be caused by development of chemoresistance subsequent to patient exposure to antileukaemic drugs [6]. Alternatively, the cause of chemoresistance may be treatment-related selection of primary drug-resistant subclones originally present at initial diagnosis [7].

Relapsed disease is generally much less responsive to treatment, and despite salvage chemotherapy regimens and haematopoietic stem cell transplantation (HSCT), most children with relapsed ALL eventually succumb to their disease [8]. Consequently, the occurrence and identification of chemoresistance in paediatric ALL is of major clinical importance, and a significant effort has been made in recent years to understand the molecular mechanisms behind non-responsiveness to treatment.

MECHANISMS OF CHEMORESISTANCE IN ALL COMMON WITH DIFFERENT CANCERS

The classical mechanism of chemoresistance in cancer cells arises via multidrug resistance (MDR), which typically affects influx or efflux of drugs through altered expression or kinetics of transmembrane transporter proteins. Although there is conflicting evidence, two drug resistance proteins currently thought to play a role in treatment outcome in paediatric ALL are P-glycoprotein (MDR1), which decreases intracellular anthracycline, mitoxantrone, taxane, epipodophyllotoxin and vinca alkaloid levels [9, 10], and lung resistance protein (LRP), which is part of a ribonucleoprotein organelle known as a “vault” thought to transport drugs away from intracellular targets [11, 12].

In addition, there is significant evidence for the pharmacogenetic impact of variations in activity of various drug-metabolising proteins on treatment responses in ALL. These variations are caused by polymorphic sequence variants in genes encoding for drug metabolising enzymes. For example, polymorphic variants of the thiopurine inactivator thiopurine methyltransferase (TPMT), glutathione S-transferases (GSTs) involved in response to prednisone, and overexpression of the folate metabolism gene thymidylate synthase (TYMS) were all shown to influence ALL treatment outcome [13-15].
The role of programmed cell death – apoptosis – in ALL chemoresistance has also become a major research focus in recent years. In response to diverse cellular signals, apoptosis proceeds through either the extrinsic or intrinsic apoptotic pathways, both of which rely upon activation of caspases for ultimate execution of substrate cleavage [16]. The extrinsic pathway involves ligation of cell surface receptors by their ligands, formation of the death-induced signalling complex (DISC) followed by caspase activation [17], while the intrinsic pathway results in stress induced cytochrome c release from the mitochondria leading to formation of the “apoptosome” complex with procaspase 9 and Apaf1 and activation of the effector caspases 3, 6 and 7 [18]. Both of these pathways are regulated by the tumour suppressor protein p53, which acts as a sequence-specific transcription factor. Protein p53 is known to be capable of activating more than 300 different promoter elements [19], and it is the coordination of expression of these genes and interaction of their individual functions that leads to the range of p53-specific cellular effects [20].

Diverse alterations in apoptotic pathways have been reported in paediatric ALL. Interestingly, although over 50% of all human cancers exhibit p53 mutational inactivation [21], less than 5% of paediatric ALL tumours actually harbour detectable mutations in this gene at ALL diagnosis [22, 23]. In contrast, hypermethylation and transcriptional silencing of p53 downstream target p21 has been found to correlate with a poor prognosis in childhood ALL [24]. Furthermore, overexpression of the anti-apoptotic protein Bcl-2 has also been shown in chemoresistant leukaemias [25, 26], whereas overexpression of the pro-apoptotic proteins Bax and anti-apoptotic protein Mcl-1 has been associated with a greater risk of ALL relapse [27, 28]. Evidence for the involvement of the extrinsic pathway in ALL chemoresistance is less clear. Upregulation of both Fas and TRAIL-R2 in response to antineoplastic drugs in ALL cells [29, 30] have been demonstrated, but these molecules appear to mediate ALL sensitivity only towards agents which cause their upregulation [29].

Finally, there have also been a number of reports of mutations in pro-survival genes in paediatric ALL, which render corresponding pathways hyperactive and can affect therapeutic responses. For example, the pro-survival gene PTEN is found to be mutated at a frequency of around 20% in childhood ALL [31] and an association between chemoresistance and loss of PTEN has been demonstrated [32]. Furthermore, constitutive activation of NF-kB pathway has been reported at a frequency of over 90% [33] in childhood ALL, whereas mutations in various pro-survival Ras genes have been reported at frequencies of 15-20% [34-37] (Table 1).

### TABLE 1. Mechanisms of chemoresistance in paediatric ALL

<table>
<thead>
<tr>
<th>Specific or common to other cancers</th>
<th>Mechanism</th>
<th>References</th>
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<tbody>
<tr>
<td>Common to other cancers</td>
<td>Transmembrane transporter proteins (MDR1, LRP)</td>
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</tr>
<tr>
<td></td>
<td>Genetic variants in drug metabolizing enzymes</td>
<td>13-15</td>
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<td></td>
<td>Defects in apoptotic pathway</td>
<td>16-30</td>
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<td></td>
<td>Defects in pro-survival pathways</td>
<td>31-37</td>
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<td>Specific to ALL</td>
<td>Specific expression of a set of genes associated with resistance to single therapeutic agents</td>
<td>38-40</td>
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<td>Specific expression of a set of genes associated with cross-resistance</td>
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<td></td>
<td>Defective gene response to DNA double strand breaks</td>
<td>60</td>
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**GENE EXPRESSION PATTERNS AND CHEMORESISTANCE IN ALL**

The advent of microarray technology in recent years has dramatically changed understanding of chemoresistance in leukaemic cells. This approach has facilitated dissection of molecular pathways involved in chemoresistant ALL. In microarray experiments, thousands of gene probes are affixed in a known configuration onto a solid matrix. Subsequently, RNA is harvested from the cell type of interest and labelled with fluorescent dyes to create a target, which can be studied for presence, abundance and identity of different genes. This target is then hybridised to the tethered probe sequences and laser light is used to excite the fluorescent dye. The resultant amount of fluorescent emission is thus a representation of the hybridisation intensity, and this gives an estimate of the relative amounts of the different gene transcripts that are present. Comparison of leukaemic samples with different biological properties can therefore identify genes deregulated in specific ALL subtypes.

Several research groups have sought to identify groups of genes, termed “transcriptional signatures”, whose expression can be directly associated with drug resistance. In their first study, Holleman and colleagues have shown that pattern of expression of a set of genes can distinguish resistance to the common chemotherapeutic agents such as Prednisolone, Vincristine, Asparaginase and Daunorubicin, and that no pattern of a single gene expression can be associated with resistance to all four agents [38]. They found that high expression of a number of genes involved in carbohydrate metabolism determines resistance to Prednisolone, high expression of genes involved in nucleic acid metabolism resistance to Vincristine, whereas the high expression of genes involved in protein metabolism determines resistance to Asparaginase (Table 1). By subsequently expanding their cohort of ALL patients and by addressing again genes associated with cross-resistance to all four agents, these authors finally identified 45 genes differentially expressed between resistant and sensitive ALL samples whose expression pattern was significantly related to treatment response [39]. These genes were involved in regulation of transcription, cellular transport and cell cycle maintenance. Using patterns of gene expression the authors
could distinguish a subgroup of ALL tumours with cross chemoresistance and inferior outcome from those which exhibited only single drug resistance. The same authors have also shown that transcriptional regulation of key apoptosis genes can be linked to cellular drug resistance and prognosis in paediatric B-lineage ALL [40]. Other microarray studies have analysed transcriptional responses to glucocorticoids but failed to define an expression signature for resistant tumours [41].

In a slightly different approach, several recent studies have used microarray analysis to ascertain underlying molecular mechanisms of chemoresistance in paediatric ALL by direct comparison between diagnostic and relapsed samples. Using paired diagnostic/relapse samples, Staal et al [42] originally identified only a small number of genes which differed in expression between the two ALL subgroups. These authors identified upregulation of signalling molecules and transcription factors involved in cell proliferation and survival at relapse rather than expected upregulation of multidrug resistance markers [42]. Subsequently, Beesley et al [43] used the same method of matched relapse and diagnostic samples and identified a much larger set of discriminative genes, many of which had been previously implicated in oncogenesis. These authors were able to successfully predict outcome in an independent cohort of 72 paediatric ALL patients. Again, no classical multidrug resistance proteins were identified as being discrepancy regulated at diagnosis. Bhojwani et al [44] used a large number of matched samples in their efforts to identify relapse-associated biological pathways in childhood ALL and found significant differences in expression of genes involved in cell-cycle regulation, DNA repair and apoptosis between diagnostic and early-relapse samples, although no discriminative gene signatures could be found for late-relapse samples [44]. The authors argued that transcriptional difference between early and late relapses may relate to the fact that patients with early relapse generally have more aggressive disease compared to patients with later relapses. The observation made by the authors that early relapses correlate with overexpression of genes involved in cell-cycle regulation was supported by the work of Kirschner-Schwabe et al, who showed that early relapse patients had a distinct expression signature of 83 genes compared with late relapses which included many cell cycle genes with function in mitosis [45].

Flotho et al used an alternative strategy to identify genes involved in chemoresistance in paediatric ALL. They analysed genes in 187 newly-diagnosed ALL samples and compared their expression profiles with MRD results at day 19 [46]. This analysis found 674 genes associated with MRD status and demonstrated that 40 genes could predict relapse in an independent cohort of 99 patients. Of these 40 genes, 14 showed independent prognostic significance. What was particularly striking in this set of data was the fact that the majority of the significant genes played a role in cell proliferation, underexpression of which appeared to relate directly to increased chemoresistance. The authors proposed the hypothesis that their finding might reflect the reduced efficacy of drugs used in paediatric ALL treatment regimens such as Methotrexate, Daunorubicin, Vincristine and Cytarabine against non-proliferating cells. Talby et al [47] applied a similar approach, by defining chemosensitivity according to the percentage of blasts present at day 21 after induction of therapy, and by analysing expression of 4205 genes in 32 patients at this time point. They used RT-PCR to further investigate differential expression of individual genes, and found that a combination of just three genes – CD34, SPI-B and BCR – was able to stratify ALL patients as either chemosensitive or chemoresistant.

Despite the enormous power of microarray information, it is important to note that the microarray approach towards chemoresistance in ALL has certain limitations. One major problem is the limited overlap between the lists of genes associated with chemoresistance generated in different studies. Indeed, none of the presented microarray studies have yet led to identification of repeatable set of genes whose pattern of expression can be used in routine clinical practice. Consequently, there have been several recent criticisms of the design of microarray studies and the need for extensive further evaluation of identified gene candidates [48-50].

The second important point is the lack of overlap between microarray data addressing chemoresistance in ALL and already established markers of ALL chemoresistance. Even in microarray experiments constructed around samples with defined resistance to various drugs, classical markers of resistance with known prognostic impact such as the P-glycoprotein genes or detoxifying enzymes such as GSTs or TYMS are very rarely detected. Whether this reflects identification of more subtle mechanisms of chemoresistance by microarray analysis or is an indicator that different approaches to candidate identification are needed remains to be determined.

**DNA DAMAGE RESPONSE AND CHEMoresistance IN Paediatric ALL**

Our laboratory has been particularly interested in ALL responses to a specific type of DNA damage – DNA double strand breaks (DSBs). Defects in responses to DNA damage are known to have a role in development of malignant disease, particularly those of lymphoid origin. For example, patients with chromosome instability syndromes such as Ataxia Telangiectasia (A-T) or Nijmegen Breakage Syndrome, both of which are caused by mutations in genes encoding proteins critical in the DNA damage response, have an increased incidence of lymphoid malignancy [51-53].

Sequence variants in the ATM gene have also been shown to have a high prevalence in childhood ALL [54]. They are particularly associated with the pathogenesis of childhood T-ALL [55]. We found that inactivation of the ATM gene in chronic lymphocytic leukaemia severely
affects the response of this leukaemia to DNA damaging agents [56, 57, 58] and remains to be determined whether ATM sequence variants can also impact on chemosensitivity of paediatric ALL.

Classes of drugs used in common multiagent chemotherapeutic regimens in paediatric ALL include DNA-damaging agents such as Etoposide and Doxorubicin, antimitabolites such as Methotrexate and Cytosine Arabinoside, mitotic inhibitors such as Vincristine and nucleotide analogues such as 6-mercaptopurine. Many of these agents induce killing by causing DNA damage in form of DSBs. Etoposide, Doxorubicin, Methotrexate, and Cytosine arabinoside have been shown to cause accumulation of the p53 protein in cells subsequent to their administration, clearly supporting a role for the induction of p53-dependent pathways in their mechanism of action [59]. Therefore, examination of the integrity of DNA damage response pathways could potentially prove useful in identifying the underlying pathogenesis of non-responsive ALL.

DNA-damaging agents and ionising radiation (IR) induce an early upregulation of p53 and its downstream transcriptional targets, including the cell cycle regulator p21, followed by later cleavage of procaspases and their downstream substrates such as PARP1. Thus, irradiation induced expression of p53, p21, PARP1 and pro-caspases 3, 7 and 9 can serve as a tool to identify ALL samples with defective responses to DNA DSBs. Using a stratification system with IR as a model to induce DNA damage in vitro, we have demonstrated that a post-irradiation expression of p53, p21 and PARP1 and pro-caspases 3, 7 and 9 delineates two major response types in paediatric B-precursor ALL: a “sensitive” phenotype which shows a reduction in PARP1 expression by 8 hours post-IR, and a “resistant” phenotype which maintains PARP1 expression at this time and up to 24 hours post-IR [60]. These results reflected patients’ responses in vivo. Sensitive patients showed a good blast clearance at day 8 or 15 of induction treatment and low risk minimal residual disease (MRD) at day 28. Furthermore, microarray comparison between leukaemias with two types of DNA damage response identified a number of genes abnormally upregulated in response to IR in resistant leukaemias, including the members of pro-survival NF-κB pathway [60]. We subsequently used these observations to devise a treatment strategy that could restore response to IR in vitro in resistant tumours. We have shown that inhibition of the NF-κB pathway can re-establish sensitivity to IR in resistant paediatric ALL tumours. Therefore, microarray analysis based on damage response classification of paediatric ALL can potentially provide new therapeutic targets.

CONCLUSION

Recent years have seen a dramatic shift in approach to study of the mechanisms behind chemosensitivity of leukaemic cells. Microarray analysis provides a way of addressing multiple and possibly interacting mechanisms of chemoresistance in paediatric ALL. More importantly, this approach allows identification of novel therapeutic targets. The number of new compounds that target specific cellular pathways is growing daily. It is, therefore, likely that future treatment strategies will improve survival of patients with ALL by specifically targeting the genes and pathways that are found to be deregulated in chemoresistant ALL.

REFERENCES

Акутна лимфобластна леукемија (АЛЛ) је најучесталије малигно бољење код деце. Упркос томе што се 80% деце с овим бољењем данас излечи, резистенција на примену терapiју код деце с АЛЛ и даље представља озбиљан клинички проблем. Због тога препознавање генез и биолошких процеса који изазивају хеморезистенцију код болесника с АЛЛ представља кључ у дизајнирању нових облика лечања и у напорима да се побољша преживљавање болесника с овим типом леукемије. Мутације у мембраним транспортерима типа P-гликохране, генетске варијације у ензимима за метаболизаме лекова и оштећења у процесу програмираних смрти ћелије представљају механизме хеморезистенције који су заједнички за АЛЛ и остала малигна бољења. Скорашње студије опште генске експресије помоћу микрочипа указали су на нове облике хеморезистенције у деци АЛЛ и, сходно томе, на нове облике лечања. Ова истраживања су показала да гени који дискримишу межу сензитивним резистентним леукемијама учествују у ћелијским процесима који регулишу ћелијски циклус, пролиферацiju и репарациjу оштећења ДНК. У овом ревијском чланку приказани су резултати студија општих генских експресија у деци АЛЛ. Такође су приказани и дискутовани наши сопствени резултати везани за одговор леукемиjsких бласта код деце с АЛЛ на двоструке прекири ДНК изазване хемотерарапијом. Приказан су и принципи класификације леукемија на сензитивне и резистентне, засновани на њиховом одговору на оштећење ДНК in vitro. Такође су дискутовани резултати који сугеришу да резистентне леукемије показују поремећен одговор NFKB ћелијске каскаде на зрачење, као и за- пажање да инхибиција овог одговора може поново да успостави сензитивност леукемиjsких ћелија.

Кључне речи: акутна лимфобластна леукемија (АЛЛ); хеморезистенција; одговор на оштећење ДНК; микрочип