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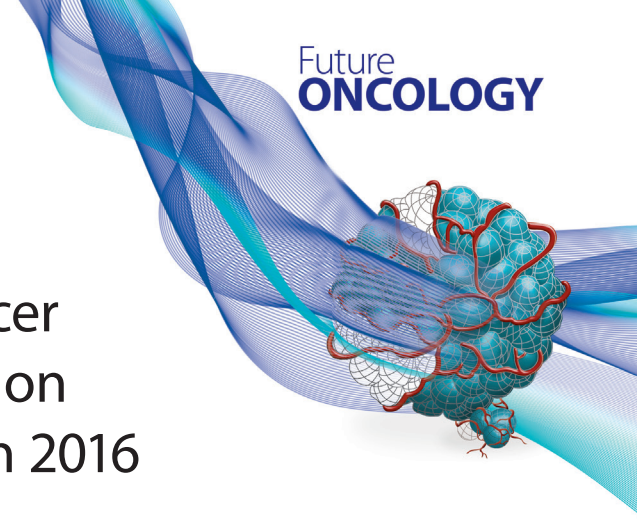


# What's new in small cell lung cancer – extensive disease? An overview on advances of systemic treatment in 2016

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Systemic therapy options for small cell lung cancer patients with extensive disease remain poor. After an initial response on first-line therapy, virtually all patients develop disease progression. For those who showed an initial response only few therapy options with low response rates are currently available. Until now, many experimental and targeted agents have failed to yield convincing clinical benefits, and new therapy options are clearly warranted for these patients. In this year's oncological congresses, several new therapy strategies, including checkpoint inhibition, showed promising results in ongoing trials. Furthermore, a potential benefit of new agents targeting DLL3, Aurora A kinase and PARP-inhibitor was reported. In this review we summarize new developments and critically highlight the most important and promising data in the relapsed small cell lung cancer disease.

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**KEYWORDS**
• alisertib • atezolizumab • checkpoint • durvalumab • extensive disease • nivolumab • pembrolizumab • rovalpituzumab-teserine • SCLC • small cell lung cancer • veliparib

Small cell lung cancer (SCLC) is a poorly differentiated neuroendocrine tumor that accounts for approximately 15% of all lung cancers [1]. This type of lung cancer is characterized by rapid tumor proliferation, early metastatic spread and responsiveness to initial therapy [2]. The majority of patients are diagnosed at advanced stages (i.e., extensive disease [ED]). The median survival among SCLC patients with ED is approximately 8 months [3]. In contrast to metastatic non-small-cell lung cancer with great advances toward personalized medicine in subgroups of patients (EGFR, ROS1, ALK) the standard treatment approach of SCLC in the first-line setting is a platinum-based combination with etoposide. However, after archiving a response upon first-line treatment, nearly all patients relapse with chemotherapy-resistant disease. In the western countries, typical treatment options after tumor progression include topotecan or the combination of cyclophosphamide, doxorubicin and vincristine. Nevertheless, second-line options usually only show modest activity and its use is restricted to patients with good performance status [4]. Thus, novel and more efficacious treatment options are urgently needed.

In the last few years, improved bioinformatics tools in combination with next-generation sequencing techniques have advanced the field of tumor genetics. These findings have resulted in a better understanding of tumor cell evolution and in the development of promising new agents targeting specific cell surface molecules [5,6]. Of these, rovalpituzumab tesirine (Rova-T), a DLL3-targeted antibody–drug conjugate, showed first promising results according to a Phase I trial in pretreated patients. Moreover, multiple immunotherapy approaches have been studied in SCLC patients. The anti-PD1 antibody nivolumab and the anti-CTLA-4 antibody ipilimumab yielded efficacy with only a low toxicity in recently presented early phase trials. Further trials are currently ongoing to

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evaluate if checkpoint inhibition in combination with targeting agents or chemotherapy might be beneficial in patients suffering from SCLC.

Herein, we aim to provide an up-to-date overview on recent developments in ED-SCLC, focusing on systemic therapy. Therefore, not only published literature but also peer-reviewed published abstracts presented at the American Society of Clinical Oncology Congress (Chicago, June 2016), at the European Society of Medical Oncology Congress (Copenhagen, October 2016) and at the World Conference on Lung Cancer (WCLC; Vienna, December 2016) are summarized.

## Immunotherapy

Immuno-oncologic treatment has incrementally changed systemic therapy within the past few years and is considered to be one of the most promising areas in cancer research. One of these new substances is the group of checkpoint inhibitors. The immune system depends on multiple checkpoints to avoid overactivation of the immune system on healthy cells. Tumor cells can take advantage of these checkpoints to escape detection by the immune system. CTLA-4 and PD-1 are checkpoints that have been studied as targets for cancer therapy [7,8].

While in 2011, only one checkpoint inhibitor was approved for melanoma (ipilimumab) [9]; in 2016, different checkpoint inhibitors have been approved for five further tumor types including NSCLC [10], head and neck cancer [11], kidney cancer [12], Hodgkin lymphoma [13] and urothelial cancer [14]. In NSCLC, the PD1 inhibitors, nivolumab [15] and pembrolizumab [16], currently became standard treatment options in second-line therapy and pembrolizumab was only very recently approved for the upfront setting in tumors harboring a high PDL-1 expression [17]. However, in SCLC none of these agents has been approved so far. Currently, several Phase II/and III studies are evaluating immune-oncologic approaches in SCLC.

Antonia and colleagues published an interim analysis of the first Phase I/II trial (CheckMate 032) in patients with SCLC progressing after at least one platinum-containing therapy [18,19]. In this Phase I/II trial, patients were allocated to three treatment arms: nivolumab 3 mg/kg every 2 weeks, nivolumab 1 mg/kg plus ipilimumab (3 mg/kg) or nivolumab 3 mg/kg plus ipilimumab 1 mg/kg. In total, 216 patients with good performance status (Eastern Cooperative Oncology Group 0–1) were enrolled and randomized to one of those three arms. The primary end point was objective response. Objective response was achieved in 10/98 (10%) patients in the nivolumab monotherapy arm, 14/61 (23%) receiving nivolumab 1 mg/kg plus ipilimumab 3 mg/kg and 10/54 (19%) receiving nivolumab 3 mg/kg plus ipilimumab 1 mg/kg. Grade ≥3 toxicities were detected in 13/98 (13%) in the nivolumab 3 mg/kg group, 18/61 (30%) in the nivolumab 1 mg/kg plus ipilimumab 3 mg/kg group and 10/54 (19%) in the nivolumab 3 mg/kg plus ipilimumab 1 mg/kg. The most commonly reported grade 3 or 4 treatment-related adverse events were increased serum lipase and diarrhea. Six patients (6%) in the nivolumab 3 mg/kg group, seven (11%) in the nivolumab 1 mg/kg plus ipilimumab 3 mg/kg group and four (7%) in the nivolumab 3 mg/kg plus ipilimumab 1 mg/kg group discontinued treatment due to treatment-related adverse events. Death from treatment-related adverse events (myasthenia gravis and worsening of renal failure) occurred in two patients who received nivolumab 1 mg/kg plus ipilimumab 3 mg/kg and in one patient who received nivolumab 3 mg/kg plus ipilimumab 1 mg/kg who died from treatment-related pneumonitis.

The median duration of response on experimental treatment was not reached for the nivolumab 3 mg/kg cohort (4.4–not reached), 7.7 months (4.0–not reached) for the nivolumab 1 mg/kg plus ipilimumab 3 mg/kg cohort and 4.4 months (3.7–not reached) for the nivolumab 3 mg/kg plus ipilimumab 1 mg/kg cohort. Sixteen patients showed prolonged response duration above 6 months.

At WCLC, updated analysis of the CheckMate 032 was presented. Two-year overall survival (OS) rates were 17% for nivolumab monotherapy and 30% in the combination arm. PDL1 expression was observed in 16% of the entire cohort. However, no predictive value could be observed when stratifying groups according to PDL1 expression status [20].

The authors concluded that both nivolumab monotherapy and combination therapy with ipilimumab showed clinical efficacy in previously treated patients with SCLC and provided an acceptable safety profile. This study gives a first insight on the potential benefit of checkpoint inhibition in SCLC.

On the basis of these promising results, several Phase III trials are currently evaluating the

role and effectiveness of checkpoint inhibitors: the CheckMate 451 (NCT02538666) study is currently investigating nivolumab in a fixed dose, nivolumab 1 mg/kg plus ipilimumab 3 mg/kg or placebo as maintenance therapy after first-line chemotherapy. CheckMate 331 (NCT02481830) on the other hand is evaluating nivolumab versus single-agent cytotoxic regimen as second-line therapy. The first results of both studies will be expected for 2018.

In 2012, Reck and colleagues published a multicenter Phase II trial analyzing ipilimumab in combination with cytotoxic therapy in the first-line setting [21]. In total, 130 patients with newly diagnosed ED-SCLC were randomized 1:1:1 to either concurrent-ipilimumab regimen (= 4 doses of ipilimumab 10 mg/kg with paclitaxel [175 mg/m$^2$] and carboplatin [area under the curve =6] followed by two cycles of placebo + paclitaxel + carboplatin), to phased-ipilimumab regimen (= 2 doses of placebo + paclitaxel + carboplatin followed by 4 doses of ipilimumab + paclitaxel + carboplatin) or to the control group (= 6 doses of placebo + paclitaxel + carboplatin). The phased-ipilimumab group showed a significant improvement of progression-free survival (PFS) compared with the control group (6.44 vs 5.26 months; HR: 0.64; p = 0.03); however, the concurrent-ipilimumab cohort failed to show a prolongation of PFS (5.68 vs 5.26 months; HR: 0.75; p = 0.11). The median OS tended to be better for the phased-ipilimumab group (12.5 months; HR: 0.75; p = 0.13) compared with the concurrent-ipilimumab (9.1 months) and placebo (9.9 months) groups; however, it did not reach significance. Altogether, ipilimumab showed clinical activity in combination with standard therapy when administered as phased regimen.

In the double-blind CA184–156 trial [22], 1132 patients were randomized to receive ipilimumab (n = 566) or placebo (n = 566) combined with etoposide and a platinum. Treatment consisted of four induction cycles of a platinum-doublet repeated every 3 weeks, with four administrations of ipilimumab at 10 mg/kg or placebo added during cycles 3–6. Subsequently, maintenance treatment with ipilimumab at 10 mg/kg or placebo was given every 12 weeks starting 9–12 weeks after induction until disease progression or unacceptable toxicity for a maximum of 3 years. The primary end point was OS. After a follow-up of 10.5 months in the ipilimumab arm and 10.2 months in the placebo arm, the median OS was 11.0 months (95% CI: 10.5–11.3) and 10.9 months (95% CI: 10.0–11.5) (HR: 0.94; p = 0.3775), respectively. Median PFS was 4.6 versus 4.4 months in the ipilimumab versus placebo cohort (HR: 0.85; p = 0.0161). An objective response rate (ORR) of 62% was seen in both cohorts. Disease control rate (DCR) during maintenance treatment was 26% with ipilimumab and 25% with placebo. The median duration of response was 4.0 months in the immunotherapy arm and 3.5 months in the placebo arm. Adverse events were more frequently detected, in the ipilimumab arm including diarrhea (25 vs 10%), neutropenia (24 vs 33%), anemia (24 vs 29%) and nausea (23 vs 16%). In general, grade ≥3 treatment-related adverse events were observed in 48 versus 45%. Discontinuation of treatment due to treatment-related adverse events could be observed in 18 versus 2%, with the most common reasons in the ipilimumab group being diarrhea (5%) and colitis (4%). Treatment-related death occurred in five patients in the ipilimumab group and two patients in the placebo group.

The authors suggest that one of the possible reasons for the failed study outcome is that without corresponding T-cell activation in the tumor microenvironment, ipilimumab monotherapy, which stimulates peripheral T-cell activation, may not be effective in achieving a sufficiently strong antitumor response.

Pembrolizumab, another anti-PD1 antibody, is currently being evaluated in several trials. In the KEYNOTE 028 trial, a Phase Ib trial, pembrolizumab was investigated in PDL-1 positive refractory SCLC. Primary end point was the ORR. In total, 24 patients with PDL-1 positive ED-SCLC received pembrolizumab 10 mg/kg every 2 weeks up to 24 weeks or until progression, and an ORR of 33.3% was reached. The median PFS and OS were 1.9 months (1.7–5.9 months) and 9.7 months (4.1 months–not reached), respectively [23]. An ongoing single-arm Phase II trial is currently investigating pembrolizumab as maintenance therapy for 12 months in SCLC patients after showing response to induction therapy. PFS is defined as a primary end point and first results will be awaited in 2017 (NCT02359019). In a further uncontrolled multicenter Phase II trial in treatment refractory SCLC, patients are currently enrolled to receive pembrolizumab in combination with paclitaxel. Primary end point is response rate (RR) (NCT02551432).

In the REACTION study, an EORTC initiated trial, pembrolizumab will be evaluated in a randomized two-armed Phase II

trial in the upfront setting (NCT02580994). Approximately 118 patients with newly diagnosed ED-SCLC are intended to be enrolled in order to receive either standard chemotherapy with platinum-doublet or chemotherapy in combination with pembrolizumab. The primary end point of this study is PFS with OS as a secondary end point. Results can be expected in 2020.

The engineered high-affinity human anti-PDL1 antibody durvalumab (MEDI4736) is currently tested in two ongoing trials. A Phase II nonrandomized efficacy trial is recruiting patients in the second-line setting for therapy with durvalumab in combination with the anti-CTLA-4 antibody tremelimumab (NCT02937818). In a further ongoing trial, the combination of the two antibodies will be tested together with radiation therapy in patients relapsing after first-line therapy (NCT02701400).

A placebo-controlled, double-blind, randomized, multicenter Phase I/III trial is going to evaluate atezolizumab, another anti-PDL-1 antibody. Carboplatin + etoposide will be tested in combination with atezolizumab or placebo in newly diagnosed ED-SCLC. In patients who show at least a stable disease after four cycles, atezolizumab will additionally be given as maintenance therapy until disease progression (NCT02763579). Primary end points are PFS and OS and final analysis is planned for the end of 2019.

Summarizing the currently available results, several PD-1 antibodies showed promising antitumor activity with manageable side effects for this highly aggressive disease. However, further research within Phase II/III trials is necessary to prove the efficacy of anti-PD-1/PDL-1 antibodies with or without other agents, and the role of biomarkers predicting response, such as PD-L1 or tumor-infiltrating lymphocytes remains widely uncertain. Despite the first promising signs of the Phase II ipilimumab trial, the following Phase III study presented in 2016 failed to show a substantial benefit in combination with standard chemotherapy.

## The antibody–drug conjugate Rova-T

DLL3 is highly overexpressed on the cell surface of both tumor cells and cancer stem cells. In neuroendocrine tumors and SCLC, DLL3 is overexpressed in 80% and inhibits cis- and trans-activation of the NOTCH pathway. In contrast to neuroendocrine tumor cells, DLL3 is not expressed on adult healthy cells and therefore antibodies targeting DLL3 might serve as a specific targeting agent in neuroendocrine carcinomas [24].

Rova-T is a monoclonal antibody–drug conjugate targeting DLL3. This conjugate linked to the antibody is a potent DNA damaging toxin called pyrrolobenzodiazepine. Using the antibody as a vehicle, this cytotoxic drug is released mainly in tumor cells [25].

The efficacy of Rova-T was investigated in the Phase I dose-escalation SCRX16–001 trial in patients with SCLC progressing on at the least one prior therapy line, including a cisplatin-based regime [26,27]. Seventy-four patients with SCLC were assigned to dose-escalation or expansion groups, followed by the investigation of the dose schedules 0.2 mg/kg every 3 weeks or 0.3 mg/kg and 0.4 mg/kg every 6 weeks of Rova-T. In the entire population a RR and DCR of 18 and 68% was detected. In 10/26 patients (38%) of the responder group, a strong DLL3 expression of >50% was observed. In the DLL3 high subgroup, the RR and DCR were even higher at 38 and 88%, respectively. Moreover, the subgroup of patients with DLL3 >50% overexpression showed a median OS of 5.8 months, median PFS 4.5 months and 1-year survival rate of 29%, compared with 2.7 months, 2.3 months and 17% for DLL3-low patients [28].

Interestingly, the efficacy of Rova-T was irrespective of the number of previous treatment lines and was comparable to the efficacy of second- and third-line therapy settings. However, no conclusions should be drawn out of these RR considering the small sample size.

The most frequent grade 3 or worse treatment-related adverse events in 74 patients with SCLC were thrombocytopenia (8 patients; 11%), pleural effusion (6 patients; 8%) and increased lipase (5 patients; 7%). Drug-related serious adverse events occurred in 28 (38%) of 74 patients. The maximum tolerated dose of Rova-T was 0.4 mg/kg every 3 weeks; the recommended Phase II dose and schedule is 0.3 mg/kg every 6 weeks.

As mentioned above, the number of patients treated in this study was small and the so far promising data should be interpreted only with caution. According to us, this study mainly serves to establish new hypotheses and further trials are clearly warranted to prove this concept. As such, the TRINITY Phase II confirmation trial is currently enrolling patients with relapsed or refractory DLL3-expressing SCLC (NCT02674568). A Phase III trial comparing Rova-T with topotecan in the second-line treatment was initiated (TAHOE

**Table 1. Ongoing Phase II/III trials in extensive disease small cell lung cancer.**

| Substance | Clinical trials phase | Design | NCT number |
|---|---|---|---|
| *Nivolumab* Anti-PD1 | Phase III (CheckMate 451) | Nivo vs nivo/ipi vs placebo as maintenance therapy after 1st line setting | NCT02538666 |
| | Phase III (CheckMate 331) | Nivo vs single agent chemotherapy in the 2nd line setting | NCT02481830 |
| *Pembrolizumab* Anti-PD1 | Phase II | Pembro vs placebo as maintenance therapy after 1st line setting | NCT02359019 |
| | Phase II (REACTION) | Platinum-eto + pembro vs platinum-eto + placebo in the 1st line setting | NCT02580994 |
| | Phase II | Pembro vs paclitaxel after 2 previous therapy lines | NCT02551432 |
| *Durvalumab* Anti-PDL1 | Phase II | Durva + treme vs chemotherapy in the 2nd line setting | NCT02937818 |
| | Phase II | Durva + treme ± radiotherapy in relapsed SCLC | NCT02701400 |
| *Atezolizumab* Anti-PDL1 | Phase III (IMpower133) | Carbo + eto + atez vs carbo + eto + placebo in the 1st line setting | NCT02763579 |
| *Rova-T* DLL3 antibody-drug conjugate | Phase II (TRINITY) | Rova-T in the 3rd line setting in DLL3-pos. SCLC | NCT02674568 |
| *Veliparib* PARP inhibitor | Phase I/II | Cisplatin + eto ± veliparib in the 1st line setting | NCT01642251 |
| | Phase II | Carbo + eto ± veliparib in the 1st line setting | NCT02289690 |

Atez: Atezolizumab; Carbo: Carboplatin; DLL3-pos.: Delta-like protein 3; Durva: Durvalumab; Eto: Etoposide; Ipi: Ipilimumab; Nivo: Nivolumab; PARP: Poly (ADP-ribose) polymerase; Pembro: Pembrolizumab; Rova-T: Rovalpituzumab-teserine; SCLC: Small cell lung cancer; Treme: Tremelimumab.

trial: NCT03061812). First patient recruitment was awaited in March 2017. Furthermore, a basket trial including DLL3-positive tumors was already initiated (NCT02709889). Only very recently, another Phase I trial combining Rova-T with cisplatin and etoposide in DLL3-expressing SCLC in the upfront setting has been launched (NCT02819999).

## Molecularly targeted therapy with alisertib

Aurora A, a member of the Aurora protein family, is a serine/threonine kinase playing a critical role in mitosis. In tumor cells the gene is commonly amplified, which leads to an overexpression of Aurora A. It seems that its overexpression activates also oncogenesis by causing genomic instability. Thus, overexpression of Aurora A is associated with a worse outcome in several tumor entities [29].

Alisertib, a selective Aurora A kinase inhibitor, could demonstrate antitumor effects in several preclinical models. Moreover, already several early trials could show efficacy in different hematological and oncological diseases [30]. At the WCLC in Vienna, Owonikoko *et al.* presented the first-in-human trial in SCLC patients [31]. After receiving first-line platinum-based chemotherapy, patients relapsing within 180 days were randomized 1:1 either to alisertib 40 mg twice daily on days 1–3, 8–10 and 15–17 in combination with paclitaxel 60 mg/m$^2$ on days 1, 8 and 15 or to placebo plus paclitaxel 80 mg/m$^2$ on days 1, 8 and 15. The primary end point was PFS. In total, 178 patients participated in this trial and a significantly prolonged PFS could be reached in the experimental arm compared with the placebo arm (2.86 vs 1.64 months; HR: 0.68; p = 0.0372). Moreover, OS (6.87 vs 5.58 months; HR: 0.73; p = 0.064) and ORR (22 vs 18%) showed a marginal benefit of the alisertib cohort, although not statistically significant.

Interestingly, a posthoc analysis could show that patients with c-myc overexpression appeared to be more responsive to alisertib than patients without c-myc overexpression (PFS: 4.64 vs 2.27; HR: 0.29; p = 0.0006).

Severe adverse events (> grade 2) could be observed more often in the experimental group than in the placebo group (67 vs 51%). Therapy discontinuation due to drug-related toxicities was detected in 15 versus 6%, respectively. Most common toxicities were observed in the combination arm stomatitis (33 vs 7%), diarrhea (59 vs 20%), neutropenia (49 vs 9%) and anemia (44 vs 20%).

In total, the Aurora A kinase inhibitor alisertib showed promising results in this Phase II trial. To prove the efficacy of this drug, sufficiently

powered trials are definitely needed. However, to the best of our knowledge no further studies evaluating alisertib in SCLC have been initiated so far. A possible explanation for that might be the increased toxicity profile detected in the alisertib arm.

## The PARP inhibitor veliparib

PARP is a fundamental enzyme in the DNA repair system. It catalyzes the transfer to target molecules, modulating in this way various necessary processes such as the chromatin structure, replication, transcription and DNA repair. Due to the fact that several tumors are dependent on PARP-mediated DNA repair for their survival, inhibiting PARP is a mechanism to stop proliferation of those cells. Several clinical trials could already show efficacy in malignancies [32].

The oral PARP1/2 inhibitor veliparib was investigated in combination with temozolomide in a double-blind Phase II trial in patients with relapsed SCLC [33]. One hundred and four patients with relapsed SCLC were randomized 1:1 to temozolomide (150–200 mg/m$^2$ on days 1–5 every 28 days) alone or to temozolimide in combination with veliparib 40 mg twice daily on days 1–7. The 4-month PFS was selected as primary end point. The 4-month PFS of both cohorts was similar (36 vs 27%; p = 0.39); however, the RR were better for the veliparib-arm compared with temozolimide monotherapy (39 vs 14%; p = 0.016). The combination therapy was well tolerated; however, more grade 3/4 thrombo- and neutropenia were observed.

Although the primary end point could not be achieved, the RR on temozolomide in combination with veliparib was significantly higher. At the WCLC 2016, data were presented which showed that high SLFN11, a response-marker to PARP inhibitors, is related to a better survival in the veliparib arm, but not in the temozolimide monotherapy arm [34]. Further posthoc predictive biomarker analyses, including MGMT, AMT and PARP, are still awaited and will hopefully clarify whether a subgroup could prolong the survival of those patients. Two studies evaluating veliparib in the upfront setting in combination with platin and etoposide have been launched (NCT01642251, NCT02289690).

## Conclusion

This review aimed to give an overview of this year's advances in the systemic treatment of ED-SCLC and intended to provide an insight in further ongoing trials. The most promising approach could be observed with immunotherapy in the relapsed tumor setting, especially in the combination of checkpoint inhibitors including the anti-PD1 antibody nivolumab and the anti-CTLA-4 antibody ipilimumab. Although encouraging survival and response improvement has been reported in those early Phase I/II trials, Phase III trials are urgently needed to evaluate the efficacy in sufficiently powered trials. **Table 1** gives a brief overview of currently

**Table 2. Overview of most relevant trials presented in 2016.**

| Study | Phase | Patients enrolled (n) | Design | ORR | mPFS (months) | mOS (months) | ≥G III° toxicities |
|---|---|---|---|---|---|---|---|
| CheckMate 032 | I/II<br>pretreated | 216 | Nivo 3 mg/kg<br>Nivo 1 mg/kg + ipi 3 mg/kg<br>Nivo 3 mg/kg + ipi 1 mg/kg | 10%<br>23%<br>10% | 1.4<br>2.6<br>1.4 | 4.4<br>7.7<br>6.0 | 13%<br>30%<br>19% |
| CA184-156 | III (double-blind)<br>1st line<br>randomized 1:1 | 1132 | Platinum + etoposide + ipi + ipi maintenance<br>Platinum + etoposide + placebo | 62%<br>62% | 4.6<br>4.4 | 10.2<br>11.0 | 48%<br>45% |
| KEYNOTE 028 | Ib<br>pretreated | 24 | Pembrolizumab 10 mg/kg | 33.3% | 1.9 | 9.7 | 8% |
| SCRX16-001 | I<br>pretreated; dose escalation + expansion | 74 | Rova-T | 18% | 2.8 | 4.6 | 38% |
| Owinokoko *et al.* | II<br>2nd line; randomized 1:1 | 178 | Paclitaxel + alisertib<br>Paclitaxel + placebo | 22%<br>18% | 2.9<br>1.6 | 6.9<br>5.6 | 67%<br>51% |
| Pietanza *et al.* | II<br>Pretreated, randomized 1:1 | 104 | Temozolomide + veliparib<br>Temozolomide + placebo | 39%<br>14% | 36%†<br>27%† | 8.2<br>7.0 | Not reported |

†PFS at 4 months.
G: Grade; Ipi: Ipilimumab; mOS: Median overall survival; mPFS: Median Progression free survival; Nivo: Nivolumab; ORR: Objective response rate; Rova-T: Rovalpituzumab-teserine.

ongoing Phase II and III trials. Those studies are currently in progress and will hopefully be presented in the next few years.

Additionally, it seems that personalized therapeutic approaches might also be beneficial in SCLC. In the last decade, many other strategies, such as inhibition of angiogenesis [35], PI3K/Akt/mTOR [36] and other tyrosine kinase inhibitors [37,38], have failed to show any benefit or only showed modest activity. Targeting DLL3, a molecule inhibiting the NOTCH-Ligand, with the antibody Rova-T has shown to prolong survival,

## EXECUTIVE SUMMARY

### Background

- The highly aggressive small cell lung cancer (SCLC) accounts for approximately 15% of all lung cancer cases.
- Although many trials analyzed different therapeutic agents, no effort could be achieved in the last decades. That is why new therapies are urgently needed.

### Immunotherapy

- Nivolumab alone and in combination with ipilimumab could showed very promising response rates (RR) in a Phase I/II trial in the relapsed tumor setting.
- In addition, the attribution of ipilimumab to first-line etoposide and platinum could neither prolong overall survival nor progression-free survival.
- However, several clinical trials investigating checkpoint inhibitors in different therapy lines are currently ongoing or will be initiated.

### Rovalpituzumab-teserine

- Delta-like protein 3 (DLL3), a molecule participating in the NOTCH signaling, is highly expressed in SCLC, but not in healthy adult tissue.
- Rovalpituzumab-teserine (Rova-T) is a monoclonal antibody–drug conjugate targeting DLL3.
- In a Phase I trial, Rova-T could show clinical activity in the second- and third-line therapy setting. Especially, in those patients with a high expression of DLL3 RR were higher than in patients with low DLL3 expression. A confirmation trial is currently in progress.

### Alisertib

- Overexpression of Aurora A activates oncogenesis resulting in a bad outcome in several malignant tumor types.
- Alisertib, a selective Aurora A kinase inhibitor, showed antitumor effects in several (pre)clinical analyses.
- In a Phase II trial, alisertib was tested in combination with paclitaxel in the relapsed tumor setting. The addition of alisertib showed a prolongation of survival and a higher RR.

### Veliparib

- PARP is a fundamental enzyme in the DNA repair system. By inhibiting PARP, proliferation of malignant cells can be suppressed.
- Veliparib, a PARP1/2 inhibitor, yielded antitumor activity in different tumor entities.
- In SCLC, temozolomide was investigated in combination with veliparib or placebo in a Phase II trial. Although the progression-free survival at 4 months was similar in both groups, higher RR were observed with veliparib.

### Conclusion & future perspective

- Checkpoint inhibitors showed a very promising clinical activity in early trials; however, large randomized Phase III trials are needed to underline the effectiveness of those therapies.
- Rova-T and alisertib are the first-targeted agents showing a benefit in relapsed SCLC. Especially for a subgroup of patients these agents could be a therapeutic option in the future.
- Personalized medicine will enter in the therapeutic management of SCLC patients in the near future. In which therapy line and for which subset of patients these substances will be approved will hopefully be answered by the ongoing or planned trials.

particularly in patients with a high expression of DLL3 detected by immunohistochemistry. To prove this concept, a Phase III trial is currently enrolling and aims to show the benefit of adding DLL3-targeted antibody–drug conjugate in the third-line therapeutic setting. Furthermore, the Aurora A kinase inhibitor alisertib yielded a prolonged PFS in patients relapsing after platinum therapy, especially in patients with a high activity of c-myc.

Altogether, to present date we do not have new standard therapy strategies for the treatment of ED-SCLC. However, these trials presented at this year's congresses showed encouraging results (for an overview see **Table 2**). After years and decades of negative trials and only modest progress, immuno- and targeted therapies might have the potential to change the landscape of the systemic treatment in SCLC. Currently ongoing Phase III trials will help to clarify the utility of these drugs and we found some reasons to have an optimistic outlook on the future. Until then patients should be encouraged to participate in clinical trials whenever possible.

## Future perspective

It is presumable that the data published and presented in 2016 will change the treatment options for patients with ED-SCLC. Especially, the outcome results achieved with nivolumab and ipilimumab again highlighted the main impact of immunotherapy in the treatment of malignancies. Thus, it is plausible that checkpoint inhibitors will receive approval in the treatment of SCLC in the next couple of years; however, in which therapy line it will be used is unclear at this point. It is evident when looking at currently planned or already initiated trials that checkpoint inhibitors will have to be analyzed in different treatment lines and in combination with diverse treatment modalities, including classical cytotoxic regimen or radiotherapy in an effort to prolong survival and to reduce tumor burden. The implementation of precision medicine as well as immunotherapy might be an essential step to prolong survival in patients with SCLC. For patients with highly expressed DLL3, Rova-T will be a very promising treatment option in the refractory tumor setting.

Although there have only been low advances in the management of SCLC patients in the last decades, there is now hope that these agents will be therapeutic options to increase the survival and the quality of life for those patients. Nevertheless, it has to be considered that sufficiently powered trials with all discussed agents have to be conducted to finally prove their efficacy in this malignant lung disease. We are optimistic that one or another drug will achieve approval as new treatment option in the future.

## Financial & competing interests disclosure

*The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

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Review

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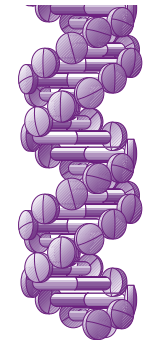
Pharmacogenomics



# Pharmacogenomics in pediatric acute lymphoblastic leukemia: promises and limitations

Despite the significant advances achieved in pediatric acute lymphocytic leukemia (ALL) treatment, adverse side effects of drugs remain a challenging issue. Numerous ALL pharmacogenomic studies have been conducted to elucidate the predisposing genetic factors for their development. Plausible pharmacogenomic data are available for the osteonecrosis associated with glucocorticoids, the neurotoxicity associated with vincristine and the cardiotoxicity related to anthracyclines. However, these data have not been fully translated into the clinic due to several limitations, most importantly the lack of reliable evidence. The most robust pharmacogenomics data are those for thiopurines and methotrexate use, with evidence-based preemptive testing recommendations for the former. Pharmacogenomics has a significant potential utility in pediatric ALL treatment regimens. In this review, gaps and limitations in this field are emphasized, which may provide a useful guide for future research design.

**Keywords:** acute lymphocytic leukemia • adverse side effects • ALL • anthracyclines • asparaginase • chemotherapy • glucocorticoids • methotrexate • pharmacogenomics • thiopurines • vincristine

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Acute lymphocytic leukemia (ALL) is the most common type of cancer in pediatrics and represents approximately 25% of all cancers in patients younger than 15 years old [1]. Advances in ALL treatment have increased the survival rate from 10% in the 1960s to 60% after the introduction of cytotoxic modalities in the 1970s. Further optimization of treatment protocols and dosing schedules in subsequent years resulted in reaching approximately 90% overall survival [2].

However, ALL continues to be a leading cause of morbidity and mortality in pediatrics [3]. The efficiently used anticancer drugs are associated with adverse side effects that interfere with treatment in 75% of ALL pediatric patients. These effects are frequently severe, especially in young age, and may lead to treatment cessation or weakens patient's adherence. Moreover, some chemotherapeutics may have delayed onset side effects or permanent sequelae [4,5]. Despite the significant advances in the field, about 1–2% of pediatric ALL patients die not because of their disease, but as a consequence of the side effects of their therapy [2]. In other estimates, treatment-related mortality was the cause of death in 3.2% of pediatric ALL patients [6].

The dramatic improvement in pediatric ALL treatment was almost entirely due to optimization of the older chemotherapeutics rather than introducing new agents. Hence, the focus of current research has shifted from finding new chemotherapeutic agents toward reducing the side effects of the

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currently used ones [2]. Pharmacogenomics (PGx) represents a promising area of research in this context. PGx studies aim at finding candidate gene variations that correlate with the interindividual variability in drug response or side effects [7]. Finding a strong association between gene variants and drug response or side effects would pave the way for preemptive testing, hence tailored therapies and individualized medicines for ALL and other treatment modalities [8].

Cancer patients can be considered as having two genomes; their constitutional genome and their tumor genome that contains all the variations of the constitutional genome together with the mutations and variations acquired during the process of tumorigenesis. Therefore, PGx studies in cancer should evaluate the effects of the constitutive variants (germline variants) as well as the effects of the acquired variants. Generally, constitutional variants affect drug transport and/or metabolism resulting in changes in treatment efficacy or side effects, whereas acquired mutations are involved in the tumor resistance to therapy [8].

In this article, mechanisms of action of different anticancer agents used for pediatric ALL are briefly described with emphasis on their main side effects. PGx studies of germline variants related to the development of these side effects are discussed with more details, focusing on what have been achieved and the gaps that need to be filled for optimum therapy.

## Overview of the molecular aberrations & diagnosis in ALL

Lymphoid cells, as other types of blood cells, originate from multipotent hematopoietic stem cells. The maturation process from the lymphoid-primed multipotent progenitors into mature lymphoid cells passes through many steps. The development of lymphoid cells involves the activation of several transcription factors and is under the control of multiple signaling pathways. Genetic defects that interrupt elements of these differentiation steps cause a range of lymphoid-cell malignancies that form the various subtypes of ALL [9].

ALL exhibits several chromosomal aneuploidies and structural aberrations as well as many deletions, amplifications and a range of point mutations [4]. It has been shown that most acquired mutations in ALL interrupt critical signaling pathways including Ras and JAK/STAT signaling, transcriptional regulation of lymphoid development and differentiation, nucleoside metabolism, epigenetic modification and cell cycle regulators [10].

Early schemes of ALL classification relied on morphology into precursor T-ALL, precursor B-ALL and Burkitt-type. However, this classification was unsatisfactory in representing the prognosis of each class [11,12]. With the advances in cytogenetic techniques including advances in karyotyping and fluorescence *in situ* hybridization, these classes were subdivided according to the recurrent chromosomal aberration. Although this approach has improved our ability to predict prognosis, the significant percentage of normal cytogenetic findings in ALL patients imposed the need for more accurate classification [12]. The recent advances in molecular techniques and mass genomic sequencing and data analysis enabled a further detailing of the submicroscopic alterations and mutations related to leukemogenesis and ALL subtype classification [11].

Pediatric ALL and adult ALL are considered as two distinct conditions. The incidence and survival rates are different in both with higher incidence rates in childhood ALL but fortunately, have better prognosis and survival rate. It has been suggested that a major difference between the two types is the origin of leukemic cells with lymphoid-committed progenitor cells being the origin of pediatric ALL leukemic cells while multipotent stem cells being the origin in adult ALL. The progenitor cells have limited self-renewal capacity and are more sensitive to apoptosis than stem cells. This may partially explain the better prognosis in pediatric ALL. Many other differences exist including the high incidence of favorable cytogenetic aberrations like hyperdiploidy (>50 chromosomes/leukemic cell) in pediatrics compared with the higher frequency of cytogenetic changes associated with a poor prognosis like the translocation *t* (9;22) in adults. Moreover, the adult leukemic cells are more resistant to anticancer drugs. This resistance could be due to differences in drug transporters expression between the two cell types. Variations in metabolic profile are another suggested cause of better response exhibited by children's leukemia cells to anticancer therapy. Consequently, pediatric and adult ALL should be considered as two distinct diseases while infancy ALL being a third category with inferior prognosis [13]. The scope of this review covers pediatric ALL that affects children between 1 and 18 years old.

## Protocols of pediatric ALL treatment

There are several approved protocols for ALL treatment with differences in dosing and may include different drug classes, especially for progressive cases. However, regardless of the protocol used, they all include three main phases lasting 2–2.5 years in total including the [1]: induction [2] consolidation and [3] maintenance phases [12].

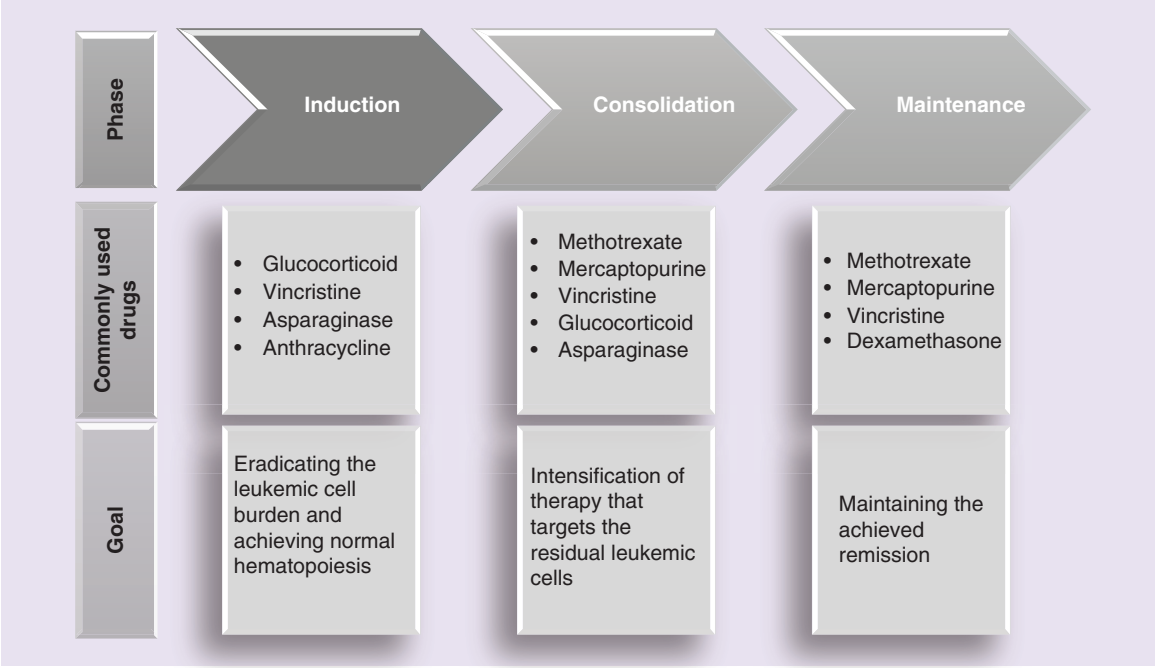


**Figure 1. Illustration of the common phases of pediatric acute lymphocytic leukemia treatment and the drugs used in most protocols.**

The induction phase aims at eradicating the leukemic cell burden and achieving normal hematopoiesis. It includes a glucocorticoid, vincristine and asparaginase with some protocols, in addition, it includes an anthracycline. Some high-risk cases may need additional drugs. The induction phase lasts for 4–6 weeks with nearly 90% of patients achieve remission. However, this is not a cure as in most cases relapse will occur without moving to the next stage of treatment [12]. The consolidation phase is considered an intensification of therapy that targets the residual leukemic cells and it includes high doses of methotrexate with a mercaptopurine. In addition, pulses of vincristine are usually given with a glucocorticoid and asparaginase [12]. The maintenance phase is the longest, spanning at least 2 years with daily doses of mercaptopurine and weekly doses of methotrexate as the main components. Pulses of vincristine and dexamethasone could be included. For some cases of very high-risk ALL in pediatrics may require an allogeneic hematopoietic stem cell transplantation before starting with the maintenance phase [12].

The phases of pediatric ALL treatment and the drugs commonly used are summarized in Figure 1.

## Adverse drug reactions of pediatric ALL therapies

Side effects and chemotherapy-induced toxicity still represent a major concern in ALL treatment. The used cytotoxic drugs have narrow therapeutic indices with some side effects being intolerable and may cause long-term sequelae, especially for the immature organs with the small body mass of children [3,8,12].

The side effects encountered are usually a consequence of the low specificity of the used drugs. These drugs are given in high doses and over a long period of time that leads to a wide range of side effects including hypersensitivity reactions, neural, cardiac, gastrointestinal, renal and hepatic toxicity, myelosuppression and osteonecrosis [3].

The body development state of children has a further effect during the treatment course. Children exhibit fluctuating levels of some drug-metabolizing enzymes like *CYP3A4*, which increases from 30–40% during the first 6 months of life to 120% of adult activity between 1 and 4 years of age. Similarly, some drug transporters, like *OATP1B1*, have lower expression levels in childhood compared with adults. These differences should be considered in anticipating side effects of treatment in diseases showing higher prevalence in childhood, such as ALL [8].

Moreover, protection of CNS from leukemic invasion in ALL patients requires the use of the intrathecal rout of administration. This rout increases the risk of developing CNS side effects of some drugs [12].

## PGx in childhood ALL

It was realized early on that a significant part of the variability in drug response in ALL patients can be attributed to genomic variants that impact drug pharmacokinetics and pharmacodynamics. Consequently, PGx

studies looking for these variations have progressed [8] and the advent of genome-wide genotyping technologies enabled the evolution of ALL PGx [14]. Pediatric ALL is an attractive platform for PGx research and clinical application for several reasons including: the presence of homogeneous and standardized treatment regimens that proved to be effective over a long period of time and in different populations; the narrow therapeutic indices of the majority of chemotherapeutic agents used; the high severity of therapy side effects, particularly in young age; some of ALL drugs including thiopurines, methotrexate and glucocorticoids (GCs) are also used for other conditions implying applicability of the obtained results for these other indications [15].

There are several approaches in PGx studies including the survey of candidate genes involved in the metabolism of the drug of interest as well as genome-wide association studies that explore very large number of SNPs in patients suffering from side effects [16]. The candidate gene approach yielded poor associations and relatively high percentage of false-positive associations. Consequently, the second approach became more favorable regarding confidence in the outcomes [15]. However, SNP-screening methodologies suffer from the limitation of missing novel or rare variants in the screened genes. Accordingly, whole-genome sequencing using next-generation sequencing platforms is currently in the forefront of choices for PGx research, albeit the cost remains a critical issue [14].

Up to date, a significant number of ALL PGx studies have been described. However, unfortunately, few of their outcomes have been implemented in the clinic. This paucity could be due to the uncertain findings of some of these studies or the slow process of information transfer from the laboratory to clinic. *TPMT* variants genotyping was the first, and the only so far, to gain regulatory approval and implementation in some countries for pediatric ALL patients [16].

## Genome-guided treatment modalities used in pediatric ALL

### Glucocorticoids

GCs, including prednisolone and dexamethasone, are essential components of chemotherapy regimens used in ALL treatment. Most protocols include prednisolone in the induction phase, and dexamethasone in the consolidation phase [17]. The cytotoxic effects of GCs are largely due to their antiproliferative activities in specific cells mediated by their binding to glucocorticoid receptors (GR) [18]. These receptors are found in two forms (GRα and GRβ) that are both encoded by the same gene but generated by alternative mRNA splicing with the GRα expressed in all cell types including lymphoid cells. Binding of GCs to GR causes a conformational change that leads to GR translocation into the nucleus where they directly activate or repress gene transcription. In the lymphoblast, they are thought to activate the apoptosis-inducing protein Bim and deactivate NF-κB and Ap1 to modulate survival negatively [19].

Major adverse reactions caused by GCs treatment include osteonecrosis, sepsis, diabetes, myopathy, hypertension or behavioral changes [20]. Osteonecrosis is one of the most common side effects and many groups attempting to find biomarkers related to its development. For example, Kawedia *et al.* performed a comprehensive analysis of factors related to osteonecrosis after GC treatment and found that among nongenetic variants, age (particularly children over 10 years old) is the strongest risk factor [21]. Moreover, they found an association between four SNPs within the *ACP1-SH3YL1* gene locus and symptomatic osteonecrosis in pediatric ALL patients treated with GCs. They proposed a role of *ACP1* in the osteoblast differentiation and bone homeostasis, but no clarification of the impact of *SH3YL1* variants was provided [21]. A more recent genome-wide association study, including 2285 pediatric ALL patients found that a polymorphism in a locus near *GRIN3A* gene was associated with an increased risk of osteonecrosis. These authors suggested that the contribution of this variant in osteonecrosis maybe due to the glutamate receptor effect on vascular supply to the bone [22].

Overall, the PGx data available about GCs response are still contradictory, and the low quality of clinical data hinder the progress in analyzing the gathered genomic data [23]. Up to date, no conclusive evidence is available for pharmacogenetic testing prior to the use of GCs in ALL or any other indication.

### Vincristine

Vincristine is a plant alkaloid that has been utilized for a long time in the chemotherapy of solid tumors as well as leukemias [24]. It can bind to β-tubulin preventing the formation of microtubules needed for chromosome alignment and separation during mitosis. Most recently, it was found to intercalate between the phosphate-sugar back bone and the histones within chromatin resulting in DNA structural alterations [25]. Without showing any myelosuppressive effect, vincristine is a reliable choice to be considered in regimens that involve myelosuppressive agents such as in the treatment regimens for pediatric ALL. Though, it was associated with other adverse events of which neurotoxicity is the major one [24].

Diouf *et al.* performed a genome-wide SNP screening in more than 300 samples from pediatric ALL patients treated with vincristine with about 22% at least showing vincristine-induced neurotoxicity. They found a correlation between polymorphisms in the promoter region of *CEP72* gene and the incidence and severity of vincristine-induced neuropathy. They examined the effect of these polymorphisms *in vitro*, by knocking down *CEP72* expression using short-hairpin RNAs (i.e., one type of siRNA), and found that the treated cells had increased sensitivity to vincristine. The authors interpreted this increased sensitivity to the role of centrosomal protein 72 in microtubule formation and the microtubule's targeting effect of vincristine. While the mechanism of neurotoxicity was not fully elucidated [26]. Other studies, which were less conclusive, found that variants in *CYP3A5* (**3*) and the vitamin D receptor were associated with peripheral neuropathy [5].

One major limitation in performing studies on vincristine-induced neurotoxicity is the difficulty to find measurable diagnosis criteria for neurotoxicity. The diagnosis depends on patient's recording of symptoms, which is a main problematic issue in pediatrics. Nevertheless, even if one strong correlation was found with a pharmacogenetic marker, more research is still needed to find the suitable vincristine doses for the different genetic variants [24].

## Anthracyclines

Anthracyclines (doxorubicin and daunorubicin) are used in many anticancer regimens as one of the mainstays of combination therapies. They exert their cytotoxic effect mainly by intercalating with DNA. Another proposed mechanism of action is by generating reactive oxygen species and hence DNA damage. The most recent explanation for anthracycline cytotoxic effect is their action as topoisomerase II inhibitors. Consequently, they prevent relaxation of the supercoiled DNA and block the replication and transcription to eventually induce apoptosis [27].

Despite their high efficacy, anthracycline use was associated with serious cardiotoxicity. These cardiovascular toxic effects are considered the third leading cause of morbidity and mortality in pediatric cancer survivors, after cancer relapse and secondary tumors. The risk of developing one cardiovascular event is increased by five- to six-times in pediatric cancer survivors compared with their siblings. This risk is progressive and delayed where it can reach up to 11-times when these survivors reach 35 years old [28]. The delayed onset of clinical manifestations in most cases complicates studying the factors related to anthracycline's induced cardiotoxicity, including the genetic factors. Though, still some patients develop early acute and subacute cardiac manifestations [29]. The mechanism of anthracycline's induced cardiotoxicity has been extensively studied with many suggestions but few firm convincing evidences. Oxidative stress induction by the accumulation of reactive oxygen species is one of the first proposed and widely accepted mechanisms. Other pathways include chelation reaction between iron and the α-ketol group of anthracyclines, toxic accumulation of anthracycline metabolites and the faulty deposition of the active drug or its active metabolites in cardiomyocytes that induce left ventricular dysfunction [30].

Several nongenetic factors were found to increase the risk of developing anthracycline's induced cardiotoxicity including high cumulative dose, younger age, female gender, black race, trisomy 21 and coexistence of cardiovascular diseases. However, interindividual differences that could not be explained by these factors alone were noticed, which suggested the presence of predisposing genetic variations [28]. Genes related to anthracycline's induced cardiotoxicity were categorized into three main groups: genes related to anthracycline metabolism pathway that can affect its biotransformation; transporter genes that affect anthracycline disposition; and genes acting on the reactive oxygen species formation [27,28]. *CBR3* is one of the most studied genes from the first group (i.e., affecting the metabolism of anthracyclines). CBR3 catalyzes the reduction of anthracyclines to the inactive hydroxyl metabolites [29]. Blanco *et al.* were the first to find an association between variants in *CRB3* and kinetics of anthracyclines, in a relatively small study [31]. Later, *in vivo* models revealed the roles of *CRB3* and other CRB family genes in anthracycline metabolism. However, clinical studies failed to show a significant correlation between variants in these genes and cardiotoxicity [29,32].

ABC transporters are among the main active proteins in exporting xenobiotics, including anthracyclines. *ABCC1* is highly expressed in the heart, and *in vivo* models proved an important protective role of this protein in cardiomyocytes from xenobiotics. Few studies evaluated the effect of *ABCC1* variants on developing cardiotoxicity after treatment with anthracyclines. Heterogeneity in treatment protocol and the parameters to identify cardiotoxicity hampered finding one strong association. Even though, a probable affect of specific *ABCC1* genotypes on the development of cardiotoxicity was suggested [33].

The third group of genes involved in the reactive oxygen species pathway includes a lengthy list of candidate genes. Wojnowski *et al.* were the first to show that variants in NADPH oxidase complex

(encoded by *CYBA*, *NCF4* and *RAC2* genes) have a significant association with cardiotoxicity induced by anthracyclines [34].

Overall, the pharmacogenetic data regarding anthracycline-induced cardiotoxicity are still inconsistent and more studies are needed, especially on the mechanisms of cardiotoxicity and the actionable gene variants in these pathways.

## L-Asparaginase

Bacterial L-asparaginase acts by catalyzing the hydrolysis of L-asparagine and depleting this essential amino acid from the tumor cells [35]. While normal cells have sufficient levels of asparagine synthetase to upregulate asparagine depletion, tumor cells are deficient in this enzyme and they, therefore, are sensitive to the asparagine deficiency that will eventually cause the death of cancer cell [36].

Two sources of asparaginase are commonly used: *Escherichia coli* derived (in native or PEGylated formulations) and *Erwinia chrysanthemi* derived. The major challenge during treatment with this enzyme is the formation of antibodies that can inactivate the drug and produce suboptimal response, and can induce a hypersensitivity reaction (occurring in 45% of pediatric ALL patients) that may lead to a life-threatening anaphylactic reaction [37].

Genome-wide association studies pointed out variants in *GRIA1* as a proposed contributor to the risk of asparaginase hypersensitivity. *GRIA1* encodes a subunit of an AMPA receptor that is suggested to play a major role during the activation of T-lymphocytes in immunoreactions. Asparaginase hypersensitivity reaction is a type I reaction that depends on T-lymphocytes activation [37].

A relatively large study by Kutszegi *et al.* confirmed the significance of the correlation between *GRIA1* variants and *E. coli* asparaginase hypersensitivity, specifically for the rs4958351 polymorphism. They demonstrate that different ALL subtypes react dissimilarly to this polymorphism. Patients diagnosed with T-ALL subtype and carrying at least one A allele at rs4958351 have shown a lower risk for asparaginase-hypersensitivity reaction compared with patients of the same diagnosis with the GG genotype. While Pre-B-ALL patients with the same alleles had a higher hypersensitivity risk. Interestingly, rs4958351 and all other found actionable polymorphisms were occurring in the intronic regions or were synonymous variants. More studies are needed to reveal the effect of these, expected to be silent polymorphisms, on the gene activity [37].

Fernandez *et al.* used another approach to define genetic variants related to asparaginase hypersensitivity. They investigated the human leukocyte antigen (*HLA*) class II alleles in 3547 pediatric ALL patient, looking for any correlation with asparaginase hypersensitivity. *HLA* class II is known to be the main contributor in hypersensitivity reactions through its activity in presenting antigens and the initiation of the immune reaction. They used microarray analysis for all SNPs occurring in *HLA* alleles and controlled other covariates that might affect asparaginase hypersensitivity (pharmaceutical preparation, tumor immunophenotype, schedule of treatment and race). A strong correlation between *HLA*-DRB1*07:01 allele and asparaginase hypersensitivity and antiasparaginase antibodies were found. They reported that this allele is associated with the production of an *HLA*-DRB1 protein with higher affinity to asparaginase epitopes, and hence hypersensitivity, in comparison to other *HLA* alleles. They emphasized the importance of pretesting for this allele and suggested designing new formulations that might be safer for patients with this allele [38].

The current recommendations in dealing with asparaginase hypersensitivity in patients treated with native *E. coli* asparaginase are to switch to the PEGylated formulation (which is known to be less immunogenic). *E. chrysanthemi*-derived asparaginase is another available option for patients who cannot tolerate the previous approach, but they need more frequent doses than the *E. coli* enzyme [39,40]. The impact of implementing pharmacogenetic testing before asparaginase treatment and the protective effect of such approach is yet to be fully evaluated and validated.

## Thiopurines

In 1953, 6-mercaptopurine (6-MP) and 6-thioguanine were approved as part of pediatric ALL chemotherapy. Since then, they proved to increase life expectancy and cure rates significantly [41]. Thiopurines (specifically azathioprine) are also used for immunosuppression in autoimmune diseases and post-transplant immunosuppression [42].

The cytotoxic effects of thiopurines depend on their structure as purine analogs. They are prodrugs and have to be transformed to their active metabolite, thio-deoxyguanosine triphosphate (TdGTP) to exert their cytotoxic effect. Two mechanisms of action were proposed for thiopurines. First, the active metabolites interfere with and inhibit the *de novo* purine biosynthesis. Second, the metabolites incorporate into the fast-replicating DNA instead of the natural purines. Incorporated TdGTPs are believed to trigger the postreplicative mismatc- repairing mechanism, but the high levels of incorporation cause the

repairing mechanism to fail. The unreparable DNA will further induce apoptosis [41,43,44].

Different enzymes catabolize the thiopurines activation process, while other enzymes compete with the former ones by metabolizing these drugs to an inactive form. Loss or compromised activity of any of the inactivating enzymes shift the balance toward an increase in the active cytotoxic TdGTPs blood levels. Toxic levels are easily approached and serious side effects, mainly myelosuppression, occur [45]. Clearly, the genes encoding the various enzyme-handling thiopurines are polymorphic with some low-activity alleles. The major enzymes with their pharmacogenetic effects on ALL are illustrated in the following sections.

## TPMT

Thiopurine methyltransferase (TPMT) is a cytoplasmic enzyme found in almost all tissues in humans. It is one of the Phase II metabolic enzymes (which mostly mediate conjugation reactions), and it is mainly involved in catalysis of thiopurine S-methylation. The importance of this enzyme in deactivating thiopurines explains the effects of its variants on the accumulation of these drugs and their toxicity and side effects. The *TPMT* gene that encodes the enzyme is a 27-kb gene consisting of ten exons [46]. The *TPMT* gene variants and their effects on thiopurines are usually used as a model for PGx studies. The correlation between genetically inherited TPMT low levels and the thiopurine toxicity was reported for the first time in the late 1980s [47]. It took more than a decade to validate and to reach to the conclusion of the need to decrease thiopurine dose for patients having reduced or non-TPMT activity as a result of their genetic makeup [48]. Up to May 2015, 37 variants in *TPMT* gene have been reported and included in the LOVD3-shared database of variants [49]. Most of these variants diminish TPMT activity, and few are associated wit47h no enzyme activity. *TPMT*3A* is considered the most common allele among Caucasian with 5% frequency, followed by *TPMT*3C* which is the most common allele in Asians. Some of the other common variants include *TPMT*2*, *TPMT*3B* and *TPMT*8* [46]. The latest recommendation from the clinical pharmacogenetics implementation consortium guidelines of 6-MP dose adjustment according to TPMT variants states that individuals with heterozygous variants who bear one nonfunctional allele (*2, *3A, *3B, *3C or *4) should be started on 30–70% of the full dose of thiopurines and individuals who have two of the aforementioned nonfunctional alleles should be started on doses reduced by tenfold or given an alternative treatment if they have a nonmalignant condition [50]. Standard dose treatment of patients with TPMT-reduced activity is associated with high risk of adverse events that might even be fatal [46].

Recently, there has been an increasing evidence of the impact of *TPMT* transcription regulatory regions in the enzyme levels and thiopurines toxicity. The promoter region of *TPMT* includes a variable number of tandem repeats (VNTRs). These VNTRs are composed of GC-rich blocks that are expected to be binding sites for transcriptional factors. These regions were divided into A, B and C repeats with the C always presents as a single copy while the number of the A and B repeats is variable. It was found that the number of A repeats is negatively correlated to *TPMT* gene expression [45,51]. Based on the architecture of these VNTR repeats, individuals can be stratified into three groups including patients with minor, intermediate or major decrease in TPMT transcription. In addition, recent evidence indicated that *TPMT* gene expression increases during the maintenance phase of ALL treatment in a VNTR architecture-dependent manner suggesting that this region should be used as a biomarker, beside the *TPMT* genotyping, before introducing thiopurine therapy [45,52,53].

As an alternative to TPMT genotyping, some studies evaluated TPMT enzyme activity measurements to predict thiopurine toxicity [54]. However, this phenotype approach had its limitations because of the other administered medications and blood transfusion that may interfere with the results. In addition, there is no consensus on uniform cut point for the low TPMT enzyme activity. A recent meta-analysis evaluated the specificity and sensitivity of *TPMT* genotyping against the TPMT enzyme activity phenotyping showed that both approaches are highly specific and that genotyping is of higher sensitivity than the enzymatic assay [55].

## ITPA

Inosine triphosphate pyrophosphatase (ITPA) is an enzyme that can hydrolyze two phosphate groups from inosine triphosphate to yield inosine monophosphate. The latter is a principal intermediate in the cellular purine biosynthesis. Patients treated with purine analogs (particularly 6-MP) will utilize the ITPA enzymatic activity in the metabolism of the drug to its inactive forms. Deficiency of ITPA, due to *ITPA* gene variants, will cause accumulation of 6-thio-inosine triphosphate, which might cause 6-MP intolerance [56].

The effect of *ITPA* variants on 6-MP toxicity has been studied following the observation that many

patients cannot tolerate 6-MP, although they carry wild-type *TPMT* alleles, an observation that was mostly reported in Asian populations.

However, the results of pharmacogenetic studies of *ITPA* are conflicting. Von Ahsen *et al.* genotyped the *ITPA* locus for the common SNPs, and studied further the architecture of the *ITPA* promoter region, in 130 blood samples from healthy individuals. They found that the rs1127354 and rs7270101 alleles are the major determinants of ITPA activity, while polymorphisms in the promoter do not affect the enzymatic activity [56]. In contrast, Ma *et al.* did not find significant differences in 6-MP tolerance between ITPA wild-type and the polymorphisms (including rs1127354) carriers, among 95 pediatric ALL patients from China [57]. Furthermore, Azimi *et al.* reported higher myelosuppression among mutant homo- and heterozygous allele carriers and have shown that these alleles are associated with greater incidence of 6-MP-related liver toxicity [58].

The effect of ITPA variants is still controversial. More studies are needed to evaluate the importance of adding ITPA testing to the list of preemptive tests for pediatric ALL patients before 6-MP treatment.

### NUDT15

Genome-wide association studies, mainly in Asian populations, described an association between a missense mutation in nucleoside diphosphate-linked moiety X motif 15 (*NUDT15*) and thiopurine-related myelosuppression. Patients carrying (rs116855232) allele (also named c.415C > T or p. R139C) were found to tolerate only 8% of the standard dose. However, the specific role that NUDT15 plays in the metabolism of thiopurines was not clear, but it was postulated that NUDT15 inhibits the incorporation of the active metabolites of thiopurine, into DNA by dephosphorylation, hence inhibiting their activity [59,60].

Two studies on Asian populations investigating the effect of *NUDT15* variants were published at the beginning of 2016. The first is by Moriyama *et al.* concluded that *NUDT15* variants affect the metabolism of thiopurines and that patients harboring the mutated allele had higher concentrations of the active metabolites and developed toxicity due to the accumulation of these metabolites [59]. In contrast, Asada *et al.* could not find a significant relation between the active metabolite of thiopurines and the *NUDT15* genotypes [61].

These contradictory conclusions can be explained by differences between both studies methodologies. Moriyama *et al.* derived their conclusion after *in vivo* and *in vitro* experiments. The *in vitro* approach involved knocking down the *NUDT15* expression followed by cell treatment with thiopurines, then evaluating the TdGTPs incorporated in DNA, which reflects more precisely the final drug effect. *In vivo*, they measured the incorporated TdGTPs in white blood cells of patients from different genotypes. Both experiments had uniform outcomes [59]. While Asada *et al.* measured the TdGTP levels in red blood cells, which represent the free active metabolites levels [61].

Regardless of the mechanism of *NUDT15* variants in inducing thiopurine toxicity, both groups found that these variants were highly correlated with thiopurine toxicity [59,61]. The rs116855232 allele is found in high frequency with 10.4% of Koreans harboring it. This is in contrast to *TPMT* gene nonfunctional variations that are considered rare in Asians in comparison to Europeans (<1.6% vs 5–10%). Thus, *NUDT15* genotypes are gaining more attention in Asian populations [60]. Inference of *NUDT15* genotypes on the data from the 1000-genome project revealed that deficiency in *NUDT15* is expected to have 22.6% prevalence in East Asians, 13.6% in South Asians and between 12 and 21% in Native American populations [59].

The mechanism of action of thiopurines and the effect of different variants in some of their metabolizing enzymes are summarized in Figure 2.

## Methotrexate

Methotrexate is a folate analog that is used in the consolidation and the maintenance phases of pediatric ALL therapy regimens. Through the inhibition of dihydrofolate reductase, it depletes the cells of tetrahydrofolate, the active metabolite of folic acid, and interferes with the purine synthesis. Its cytotoxic action depends upon the expression of many enzymes in the folate pathway including MTHFR [62–64].

The methotrexate absorption, transportation, entrance into the hepatic circulation and excretion through glomerular filtration are all carried by active protein transporters. Within cells, methotrexate is converted into methotrexate polyglutamate, which is an active metabolite with higher efficiency than the parent drug. SNPs that affect the production of any of the proteins acting in the methotrexate pathway are thought to be behind the interindividual differences in methotrexate response and side effects [63].

About 68–75% of interindividual variability in methotrexate kinetics is supposed to be due to genetic differences [64]. The adverse events during methotrexate course include gastrointestinal toxicity, hepatic and renal toxicity, neuropathy and myelosuppression. Severe adverse events can lead to cessation of therapy

that in itself has a negative effect on survival in addition to the sequelae of the toxic events [62]. Many studies were designed to find out the effects of variants in methotrexate pathway key genes on interindividual differences. The results were of little significance in most cases due to small study cohorts, heterogeneous protocols and replication failure [64].

Germline variants in the *MTHFR* gene affect the folate pools and consequently may have a significant role in methotrexate activity, besides the role of MTHFR enzyme in methotrexate metabolism. Numerous studies evaluated the effects of *MTHFR* variants on methotrexate toxicity and pediatric ALL relapse. Two genotypes (c.C677T and c.A1298C) were suggested as being correlated with methotrexate toxicity [65]. A recent study by Kałużna *et al.* found that the c.667T allele is associated with lower methotrexate clearance. Patients from the same study who had a haplotype that combines c.667T with c.1298A had significant higher methotrexate toxicity, in contrast to the 667C-1298A haplotype that showed a protective effect from methotrexate toxicity [66].

SLCO1B1 is a carrier mainly expressed in the liver and acts as a transporter of many endogenous compounds and drugs. Treviño *et al.* were the first to report a correlation between *SLCO1B1* and methotrexate following a genome-wide association study. In this study, 398,699 SNPs were tested against methotrexate clearance in 434 newly diagnosed ALL pediatrics. The strongest association was annotated to the transporter gene *SLCO1B1* [67]. Later studies proved this correlation in different populations. Among ten polymorphisms, rs11045879 CC in *SLCO1B1* was the only one with significant correlation with methotrexate plasma concentration, which proved to be an objective marker of toxicity in the same study [68]. More recently, similar association has been reported from a study in China and the AA genotype or variant rs4149081 [69].

The *ABCB1* encodes for a membrane transporter glycoprotein. It has been extensively studied against different chemotherapeutic agents known to be substrates of this transporter, including GCs, anthracyclines and vincristine. Few studies evaluated the effect of these variants against methotrexate. Gregers *et al.* found that plasma levels of methotrexate did not differ significantly among different patient's groups defined by their *ABCB1* genotype. However, the 3435CC genotype was associated with increased liver toxicity [70].

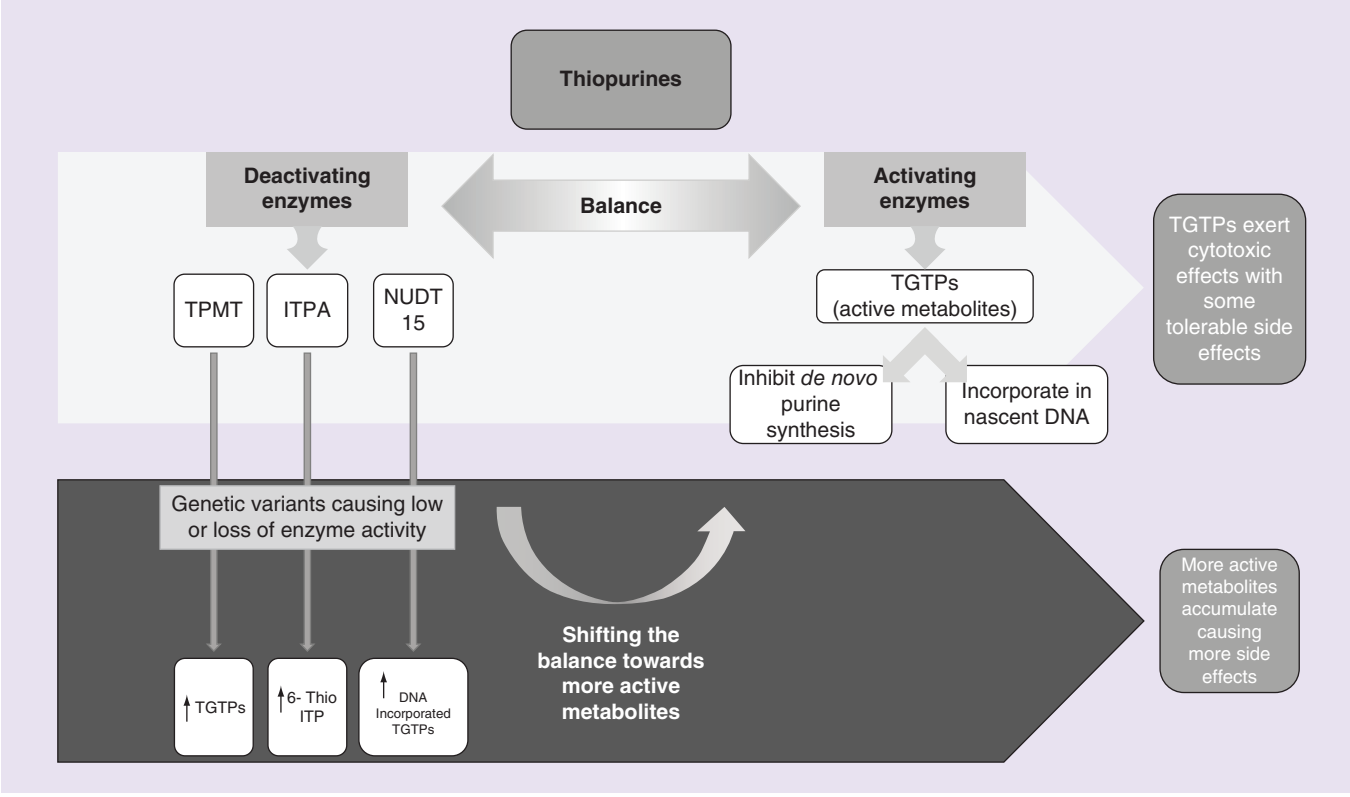


**Figure 2. The significant pathways of thiopurines activation and deactivation in pediatric acute lymphocytic leukemia, and the effects of some genetic variants.**

ITPA: Inosine triphosphate pyrophosphatase; NUDT15: Nucleoside diphosphate-linked moiety X motif 15; TGTP: Thioguanine triphosphate nucleotide; TPMT: Thiopurine methyltransferase.

Table 1. Common drugs used in pediatric acute lymphocytic leukemia treatment with examples of their common side effect and the genes known to be associated with their development.

| Chemotherapeutic agent | Side effect | Gene | Ref. |
|---|---|---|---|
| Glucocorticoids | Osteonecrosis | – *ACP1-SH3YL1*<br>– *GRIN3A* | [21,22] |
| Vincristine | Neuropathy | – *CEP72*<br>– *CYP3A5* | [5,26] |
| Anthracycline | Cardiotoxicity | – *CRB3*<br>– *ABCC1* | [31,33] |
| Asparaginase | Hypersensitivity | – *GRIA1*<br>– *HLA-DRB1* | [37,38] |
| Thiopurines | Myelosuppression | – *TPMT*<br>– *ITPA*<br>– *NUDT15* | [47,56,60] |
| Methotrexate | GI, renal and neural toxicity | – *MTHFR*<br>– *SLCO1B1*<br>– *ABCB1* | [66,67,70] |

ITPA: Inosine triphosphate pyrophosphatase; TPMT: Thiopurine methyltransferase.

Despite a large number of studies devoted for methotrexate pharmacogenetics, there is no definite recommendation for any pharmacogenetic testing before methotrexate treatment and therefore it is still an important topic for research.

## MiRNAs may play a role in the toxicity of pediatric ALL treatment

PGx studies of genes associated with pediatric ALL treatment side effects were mostly focusing on variants in genetic-coding regions and, to a lesser extent, their promoters. However, a recent novel approach considered the effects of miRNAs in regulating the expression of these genes [21]. miRNAs are a type of small-noncoding RNAs that are produced and processed at the nucleus and exported to the cytoplasm where they recognize certain mRNAs inhibiting these targets or degrading them leading to downregulation of their expression. The biogenesis and action of miRNAs are controlled by genes of the miRNA processing machinery. These latter genes have been under investigation lately as proposed effectors in regulating genes involved in chemotherapeutics' transport or metabolism [71].

In this context, López-López *et al.* found, and for the first time, an association between rs639174 in *DROSHA* and toxicity related to methotrexate during childhood ALL treatment. *DROSHA* is known to encode RNAse III enzyme involved in maturation steps that lead eventually to the formation of miRNA [72]. More recently, Iparraguirre *et al.* investigated all known SNPs in miRNAs thought to affect methotrexate transportation and found three SNPs in miR-5189, miR-595 and miR-6083 that might affect methotrexate transporters and blood levels in pediatric ALL patient [73].

Predicting the impact of miRNA processing defects on a single drug toxicity mechanism is not an easy task because each miRNA can affect more than one mRNA and many miRNAs can target the same mRNA. Despite the complexity of this nascent field of research, it holds promise for contributing to our understanding of the interindividual differences in toxicity in pediatric ALL therapy [71].

## Conclusion & future perspective

Understanding the molecular pathways of side effects' development enabled the screening for pharmacogenetic biomarkers in candidate genes. On the other hand, genome-wide screening approaches provided more trusted associations between genetic variants and side effects developed during or after childhood ALL treatments. Table 1 summarizes examples of PGx research outcomes in pediatric ALL treatment-related side effects.

Although a huge body of PGx research in pediatric ALL have already been achieved, much more is still needed for the identification of the most relevant and actionable variants. The advent of next-generation sequencing and genotyping techniques and data analysis are expected to shorten the time needed between recognizing a gene–drug correlation and its approval of clinical application.

It is important to avoid the deficiencies in the previous studies while designing new experiments. Small sample sizes limited the outcomes in many PGx studies. Larger sample sizes can be achieved through establishing large international collaborations.

No doubt that PGx has a significant potential utility in improving outcomes in pediatric ALL treatments. The main challenge now is increasing the confidence in research outcomes and enhancing the experience in interpretation of PGx testing results.

### Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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## Executive summary

- Acute lymphocytic leukemia (ALL) is the most common type of cancer in pediatrics, and adverse side effects interfere with treatment in about 75% of cases.
- Pharmacogenomic studies gained increased attention in pediatric ALL due to extended periods of treatment and low therapeutic indices of cytotoxic drugs.
- Glucocorticoids (GCs) are used for their antiproliferative activity.
- Variants in *ACP1-SH3YL1* gene locus and *GRIN3A* are proposed as candidate biomarkers for GC-related osteonecrosis. Though, no evidence is available for pharmacogenomic testing for GCs.
- Vincristine exerts its cytotoxic effect through preventing microtubule formation and its intercalation in DNA.
- *CEP72, CYP3A5* (**3*) and *VDR* are genes that may be related to vincristine-induced neurotoxicity.
- DNA intercalation and reactive oxygen species generation are the primary cytotoxic effects of anthracyclines, and cardiotoxicity is their major side effect.
- *CRB3, ABCC1* variants are among the several found variants correlated with anthracycline-related cardiotoxicity.
- Asparaginase depletes tumor cell from asparagine.
- *GRIA1* and some HLA class II variants were found to be correlated with *Escherichia coli* asparaginase hypersensitivity. However, switching to other formulations is the standard practice currently to deal with asparaginase hypersensitivity.
- Tiopurines are purine analogs that interfere with purine synthesis and incorporate in newly synthesized DNA.
- *TPMT* genotyping is one of the first approved pharmacogenetic testing that proved to be effective. NUDT15 is another important biomarker, especially in Asian populations.
- Methotrexate is a folate analog that depletes the cells of tetrahydrofolate and interferes with purine synthesis.
- Variants in *SLCO1B1* and *ABCB1* are proposed as effectors in methotrexate toxicity, but finding other biomarkers is still a hot topic for research.
- Variants in miRNA processing pathways may contribute to several drug toxicities in pediatric ALL treatment.
- Evidently, pharmacogenomics research has an apparent implication in the improvement of pediatric ALL treatment outcomes.

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Melanoma Management

INTERVIEW

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# An interview with Giuseppe Argenziano: an insight into the field of dermoscopy

**Giuseppe Argenziano* speaks to Sebastian Dennis-Beron, Commissioning Editor:** Giuseppe Argenziano is Full Professor and Head of the Dermatology Unit at the University of Campania, Naples, Italy. His main research field is dermato-oncology. He is an author of numerous scientific articles and books concerning dermoscopy, a new technique improving the clinicians detection of benign and malignant skin tumors. As a coordinator of a Skin Cancer Unit, he has established a successful tertiary, multidisciplinary, referral center particularly devoted to the diagnosis and management of patients with skin tumors. Over the past 20 years he has supervised over 100 foreign students and 40 residents in dermatology, established scientific collaborations with more than 200 colleagues from more than 30 nations, and organized more than 50 national and international scientific activities, courses and conferences (such as the Consensus Net Meeting on Dermoscopy and the First Congress of the International Dermoscopy Society). He is co-founder and past president of the International Dermoscopy Society; project leader for the development of a high diagnostic technology oncologic center at the Arcispedale Santa Maria Nuova IRCCS in Reggio Emilia; faculty member of the Master of Science in Dermoscopy and Preventive Dermato-oncology and has undertaken a short course in dermoscopy, two e-learning courses by the Medical University of Graz and by Cardiff University, respectively; and member of the Editorial Board of the Journal of the American Academy of Dermatology. Professor Argenziano has authored more than 400 full scientific articles and produced landmark primary publications and books in the field of dermoscopy. Over the past 20 years, he has been invited as speaker and/or chairman in more than 500 national and international conferences in the field of dermatology. His combined publications have received a sum total of more than 8000 citations with an h-index value of 46.

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**Q** What inspired you to develop a career in dermatology?

I have a very strong family influence; my father is a dermatologist and my grandfather was a dermatologist. So in some way you could say I was attracted by my family's history in the field. My work in melanoma specifically, began quite early in my career; I was in contact with patients with melanoma and other skin cancers as my medical degree thesis was on these particular issues, and this is why I was very early attracted by this topic.

**Q** Did you have any colleagues that were strong influences in your career?

There are two main people that influenced my professional development. One is, first, my father who taught me a lot and second was P Soyer (University of Queensland,

**KEYWORDS**

• dermoscopy • melanoma • skin cancer

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Australia). Soyer, a colleague in dermatology, who worked in Graz, Austria for many years, is now chairman of the Dermatology department in Brisbane, Australia. He was basically my mentor from a scientific perspective because he introduced me to the science of dermoscopy and melanoma. We spent many hours studying together.

**Q What would you consider your biggest professional achievement to date?**

There are two main experiences I could mention: those related to my patient care and second, those related to my teaching. In terms of my teaching, where I count my main achievements, I can recollect an experience a few years ago where I taught a course on recognizing melanoma. Following the course, a colleague went home and she was able to recognize a particularly difficult melanoma to diagnose. This colleague then explained that because of this course she was able to recognize this lesion as melanoma where she was not able to do so before hand. This is essentially what I try to achieve every day. Not only do I work with patients and try to do the best that I can for them, but also crucially, I aim to improve the quality of my colleagues' professional work and in turn be able to potentially influence the diagnosis of many more patients, on top of the ones I see face to face.

**Q You recently departed as President of the International Dermoscopy Society. During your time there, how did you see the field of dermoscopy grow?**

Dermoscopy has grown a lot in the last 15–20 years; I was acting president of the International Dermoscopy Society for 6 years and during those 6 years, my main goals were to improve the spread of the technique worldwide. When I started as president we had 2000–3000 members, which has now increased to upward of 8000. Within the society, we published a lot of papers in the field of dermoscopy and we promoted a lot of events, such as conferences and congresses to increase access and understanding of the field. As a result, today in many countries, dermoscopy is a basic tool that all dermatologists use in their practice. In the last 10 years, we have been able to bring thousand dermatologists into the field of dermoscopy which in my view is one of our greatest advancements

There are two real important landmarks in the last few years, which I would say were two main consensus meetings, one consensus published in 2000 and another which was recently published in 2016. Consensus meetings are guidelines which all dermatologists refer to; in the consensus of 2000, we were able to establish a basic methodology on how to use the dermatoscope, breaking down the methods and algorithms used to reach a particular diagnosis.

The main goal of the 2016 consensus paper was to bring together the analytic terminology and the metaphoric terminology to develop a more detailed description of the various criteria we can see through the dermatoscope. As a result, we were also able to get rid of redundant terminologies which were published in the last few years and, therefore, have a more synergistic group of criteria.

**Q Your research focuses on the use of developing more accurate methods for the early recognition of melanoma. How important is the use of dermoscopy not only in melanoma diagnostics, but also in its management?**

The main goal of dermoscopy is its use as a diagnostic tool, but it can also be used for the management of melanoma. We are able to use dermoscopy for monitoring a lot of skin cancers, which are treated with nonsurgical methods. For example, we use it a lot when we use topical treatments to treat basal cell carcinoma, since this is not a surgical procedure. Using the technique, you can verify that the use of the cream was able to completely remove the basal cell carcinoma. This is one example in which we can use dermoscopy in the field of management.

This is also true in all dermatology patients, not only skin cancer patients. Dermoscopy can be used as a diagnostic technique for a variety of dermatological issues outside skin cancer, such as psoriasis and eczema.

**Q There is a lot of discussion regarding the use of teledermatology in clinics. How would you describe this emerging field & how important is its impact on patient care?**

Teledermatology is a very interesting technique that has developed a lot in the last 20 years, with many papers being published in that time. However, it is only able to help if we are dealing with a country where there are very few dermatologists. For example, in Italy there are 4000 dermatologists. As a result, no patient will ask for a consultation via teledermatology, as there is simply no need for it.

In countries like Australia or in the UK, where there are fewer dermatologists, it is extremely important to add this technique to increase patient access. Dermatology is a perfect field for teleconsultation, because in the vast majority of cases you are able to diagnose and manage the patient with just a picture.

Paradoxically, teledermatology is much simpler in the general dermatology field, than in the skin cancer field. In skin cancer, teledermatology does not really suffice, as although you have the morphology of the lesion in an image, you still need to grasp how rest the of the patient's lesions look, understand the patient skin type and have an idea of their patient history. But in general dermatology, teledermatology is extremely useful; 5% of my daily work involves consulting with my patients via WhatsApp©. Many patients send pictures of their lesions and you can easily solve very benign problems, usually in a minute, saving a lot of time.

There is the potential for teledermatology to have a strong impact if used correctly. Aside from diagnosis, you can monitor the patient treatment, for example, through a very common way of exchanging images between the patient and consulting dermatologist, to ensure the treatment is working.

**Q** As the field of melanoma rapidly evolves, how important is it for clinicians to collaborate while managing the disease, from diagnosis through to treatment?

Melanoma and skin cancers in general, are multidisciplinary diseases. As a dermatologist, you act as the center and you work with various other disciplines in order to treat and manage the patient.

As dermatologists we have to understand the latest developments in melanoma therapy, since we are connecting the patients from one specialist to the other. We need to be aware of the latest possibilities and guidelines available. We must collaborate with other specialists, like the pathologists, the radiologists and the oncologists, to determine the needs of the patient and work around them.

The workflow is quite straightforward; if you see a lesion suspected to be melanoma, you need the pathologists to get the final diagnosis. The patient is then sent to the surgeon for the sentinel node biopsy, and then if there is something suspicious discovered by the dermatological follow-up, you have to decide the plan of action, be it surgical or medical, together with the surgeons and oncologists.

**Q** What barriers stand in our way in terms of expanding the field of dermoscopy?

The known barriers are still the economic ones, with clinicians claiming that they do not have a dermatoscope in their office as it is too expensive. This should not be an issue as dermatoscopes are now approximately US$400. The biggest barrier, I believe, is the lack of training in the field, though nowadays in many countries, there are teaching programs on going where dermatologists are being trained in the technique.

I would not say that there are really important barriers in terms of moving forward and spreading the technique and in my view, in another 10 years, dermoscopy will be a standard in all dermatological consultations. It will be like ophthalmologists not using their tools to look into the eyes – it is simply not possible.

**Q** What direction do you see the field taking in the future?

There is still much to do. You can see from the number of publications; every year there is an increasing number of papers being published in dermoscopy, which means the field is still expanding.

Though basic aspects of the field have already been established, there are of course some areas that still need to be improved, such as recognizing facial melanomas and amelanotic melanomas which are still very difficult lesions to diagnose.

There will be more and more papers published in the field of general dermatology, as each of the dermatological conditions has its own dermoscopic counterparts. It means, in my view, that the future of dermoscopy will focus additionally on the nontumoral field in general dermatology, other than skin cancers.

## Disclaimer

*The opinions expressed in this interview are those of the interviewee and do not necessarily reflect the views of Future Medicine Ltd.*

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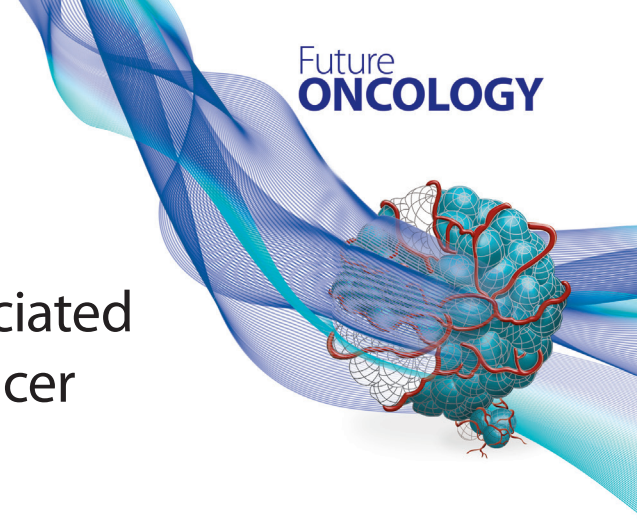

# High expression of COX5B is associated with poor prognosis in breast cancer

Shui-Ping Gao‡[1,2], He-Fen Sun‡[1,2], Wen-Yan Fu[1,2], Liang-Dong Li[1,2], Yang Zhao[1,2], Meng-Ting Chen[1,2] & Wei Jin*[1,2]

**Background:** Cytochrome c oxidase subunit VB (COX5B), a subunit of mammalian COX, takes roles in COX assembling and functions. Online database predicts high *COX5B* transcription may be associated with worse disease-free survival (DFS). However, the clinical implications of COX5B in breast cancer remain unclear. **Methods:** We carried out immunohistochemistry on tissue microarrays of 244 patients with invasive ductal breast carcinoma to detected COX5B expression. **Results:** Our results suggest that COX5B protein level might be associated with tumor size. COX5B overexpression indicated a worse DFS ($p < 0.05$) in breast cancer. Furthermore, high COX5B expression may act as an independent factor for worse DFS in breast cancer. Conclusions: Cumulatively, our findings suggest that COX5B might serve as an important prognostic factor for breast cancer.

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**KEYWORDS**
• breast cancer • COX5B
• disease-free survival • IHC
• mitochondria

Breast cancer, with high incidence and mortality for recent decades, has been one of most dangerous cancers and poses a serious threat to female health in the world [1,2]. As is well known, breast cancer is a widely heterogeneous disease, which can be classified into four subtypes using immunohistochemistry (IHC). The Luminal A-like subtype is positive for estrogen receptor (ER) or progesterone receptor (PR), and negative for HEGF receptor 2 (HER2) and less than 14% of the cells are Ki-67-positive. The Luminal B-like subtype is ER or PR positive, with HER2-negative or positive and ≥14% of the cells are Ki-67-positive. HER2-positive subtype means the expression of HER2 is positive but negative expression of ER and PR. Triple negative breast cancer is defined as absence of ER, PR, HER2 in tumor [3–5].

Recently, mitochondria, the double membrane and semiautonomous organelles, are now recognized as one of potential factor with cancer progression as they provide a place for various essential physiological activities, such as reactive oxygen species production, ATP turnover, oxidative phosphorylation [6–8]. The growing evidences show that mitochondrial dysfunction has been shown to be associated with many human disorders, such as metabolic diseases, aging, nervous system diseases [9], cardiac disorder [10] and cancer progression [11].

Cytochrome *c* oxidase (COX), also known as respiratory chain complex IV, is an important structural component of the inner membrane of mitochondria [12]. It transfers electrons from reduced cytochrome *c* to oxygen molecules, pumping four protons out of mitochondria and driving FO-F1

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ATP synthase at the same time [13]. Therefore, COX is considered as a key variable in determining mitochondrial content and activity [14,15]. And COX5B (VB), together with other 12 subunits, comprises structures of complex IV. It has been reported that COX5B has a function in regulating or assemble other subunits in this complex [16–18]. In the previous studies, mRNA expression of *COX5B* was reported to be increased in skin cancer and the protein level of COX5B was also showed to be elevated in prostate cancer ($p < 0.05$) [19,20].

In our previous study, we found COX5B is elevated both in breast cancer tissues and cells lines, and loss of COX5B could inhibit cell proliferation or invasion and promote cell apoptosis. The predictable survival outcomes based on mRNA showed that there exist difference between high and low *COX5B* expression. However, the clinical value of COX5B protein by IHC remains unclear in breast cancer. In order to assess the prognosis, IHC was performed on tissue microarray (TMA) to determine whether COX5B can influence the patients' survival in breast cancer [21].

## Materials & methods

### • Patients & samples

The sample set containing 250 patients diagnosed as primary breast cancer was collected from Fudan University Shanghai Cancer Center (Shanghai, P.R. China) between August 2001 and March 2006. All patients with invasive ductal breast carcinoma (IDC) have signed the informed consent document before undergoing surgery including breast conservation surgery or mastectomy with axillary lymph node dissection surgery. And postoperative adjuvant therapies were given to patients according to standardized guideline-based chemo, hormone and radiation therapies. Patient follow-up statuses were regularly followed and the data were last revised in September 2013. The follow-up period was defined as the time from surgery to the last observation for censored cases or relapse for complete observations.

### • TMA construction

The tissue samples were fixed in formalin and embedded in paraffin postoperation. The representative regions of each tumor were identified referring to hematoxylin and eosin and punched in cylindrical form and then transferred on the special receiver slides. The whole set of TMA contains five sections or blocks according to ER, PR, HER2 or Ki-67 coloration, and each sections have 50 patients tissues. A duplicate core of the same tumor from different areas was made to increase the precision of experiment.

### • Immunohistochemistry

The TMA slides were dewaxed with xylene for 10 min, gradually hydrated with a gradient series of ethanol and washed with phosphate-buffered saline. TMAs were treated with peroxidase-blocking solution for 15 min to consume the endogenous peroxidase and then washed two-times with distilled water to rinse the remnant. During the process of antigen-retrieval, the cylindrical tumor cores were first boiled for 5 min in pH 6 citrate buffer and extended 5 min after turning-off, then allowed to slowly cool down under the condition of ambient temperature. Next, the TMAs were blocked with 10% normal goat serum for 20 min at room temperature and were incubated with mouse monoclonal anti-human COX5B antibody (Santa Cruz; 1:100) at 4°C overnight. Following washes with 10-min phosphate-buffered saline for three-times, the TMAs were incubated with secondary antibody for 60 min at room temperature. The immunoreactive products were visualized during the catalysis of 3,3-diaminobenzidine by horseradish peroxidase and stop by extensive washings when stained with optimum brown. Finally, the TMAs were counterstained with hematoxylin and dehydrated in an ascending ethanol series before clearing with xylene and mounting under a coverslip.

### • IHC score evaluation

Staining was visualized using colorimetric detection with 3,3-diaminobenzidine. The semiquantitative classes were used to describe the staining distribution. The staining intensity was evaluated as follows: 1, weak; 2, moderate; and 3, intense. And the staining distribution was determined a value from 0 to 4 as follows: 0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, >75% [22]. Then, the staining distribution and the staining intensity were multiplied to obtain the final score for each case. The patients corresponding to score under three points were considered to have low COX5B expression, and those with a score of 4–12 were considered to have COX5B high expression.

### • Statistical analysis

Disease-free survival (DFS) was used to assess COX5B clinical value in this study. During

period of follow-up, any patient has a recurrence of disease at a local, regional or distant site for first time that is considerate as DFS events. First, the correlations between the clinical–pathological parameters and COX5B expression were tested using the $\chi^2$ test. Survival outcomes were assessed by Kaplan–Meier method and compared between the low and high COX5B group by log-rank statistics. Univariate and multivariate Cox proportional hazard models were used to determine the associations of the clinical–pathological parameters with survival outcomes. All p-values in this study are two-sided, and p-values less than 0.05 were considered significant. The SPSS software, version 20.0 (IBM Corporation, NY, USA), was employed to execute statistical analysis. All analyses were based on the observed data with the assumption that missing data were missing completely at random.

## Results

### • Patient characteristics & follow-up data

A total of 250 primary breast cancer samples with follow-up data were collected at our cancer center. The pathological pattern was invasive ductal carcinoma. During several IHC processes, some tissue cores experienced their loss due to some operational reasons like sliding off during antigen retrieval. Finally, 244 cases were enrolled to clinical statistical analysis. And the basic clinicopathological characteristics of patients in this study were summarized in **Table 1**. As shown in this table, a total of 118 (48.36%) patients were under age of 50 years and 126 (51.64%) patients were over 50 years or older. And 108 (44.26%) patients were premenopausal, 136 (55.74%) patients were postmenopausal. The proportions of positive expression of ER, PR and HER2 were 42.21, 25.82 and 40.16%, respectively. With respect to tumor size, bigger than 2-cm tumors were noted in 131 (53.80%) patients and 111 (45.40%) patients below or equal to 2 cm. A total of 93 patients (38.11%) were lymph node positive and 77 patients were lymph node negative (37.56%). Triple-negative breast cancer (TNBC) accounted for 40%, while another 59.2% was not-TNBC proportion.

### • Correlations of COX5B expression & other clinical–pathologic characteristics

After staining COX5B by IHC, we found it was mainly confined to the cytoplasm of the mammary carcinoma cells **(Figure 1)**. The correlations between COX5B and clinical parameters were investigated and shown in **Table 2**, and the expression of COX5B was associated with tumor size status (p = 0.044). However, no significant associations were observed with other tumor parameters such as age, menopause status, etc.

### • High COX5B expression indicates worse clinical outcome in breast cancer

After a mean follow-up time of 98 months, 55 of the 244 patients suffered disease recurrence. Then, IHC were performed to detect COX5B expression in 244 patients' tissues, which help

**Table 1. Clinical–pathological characteristics of the study cohort.**

| Clinical–pathological characteristics | Case number (n) | Percentage (%) |
|---|---|---|
| Age (mean: 52.02, SD: 9.462, median: 51, range: 29–85): | | |
| – ≤50 | 118 | 48.36 |
| – >50 | 126 | 51.64 |
| – Unknown | 0 | 0.00 |
| Menopausal status: | | |
| – Premenopausal | 108 | 44.26 |
| – Postmenopausal | 136 | 55.74 |
| – Unknown | 0 | 0.00 |
| ER status: | | |
| – Positive | 103 | 42.21 |
| – Negative | 141 | 57.79 |
| – Unknown | 0 | 0.00 |
| PR status: | | |
| – Positive | 63 | 25.82 |
| – Negative | 179 | 73.36 |
| – Unknown | 2 | 0.82 |
| HER2 status: | | |
| – Positive | 98 | 40.16 |
| – Negative | 146 | 59.84 |
| – Unknown | 0 | 0 |
| Tumor size (cm): | | |
| – ≤2 | 111 | 45.40 |
| – >2 | 131 | 53.80 |
| – Unknown | 2 | 0.82 |
| Lymph node status: | | |
| – Positive | 93 | 38.11 |
| – Negative | 149 | 61.07 |
| – Unknown | 2 | 0.82 |
| Grade: | | |
| – I + II | 169 | 69.26 |
| – III + IV | 73 | 29.92 |
| – Unknown | 2 | 0.82 |
| TNBC: | | |
| – Yes | 100 | 40 |
| – No | 148 | 59.20 |
| – Unknown | 2 | 0.8 |

ER: Estrogen receptor; HER2: HEGF receptor 2; PR: Progesterone receptor; TNBC: Triple-negative breast cancer.

to evaluate the clinical significance of COX5B in breast cancer. According to the intensity and distribution of COX5B staining, patients were sorted into the higher and lower groups. The log-rank test was employed to examine difference of DFS curve between two groups in terms of COX5B expression, the results showed that patients with higher COX5B expression had worse DFS than those with lower COX5B expression ($p < 0.05$). We also found significance clinical value in subtype of Luminal A and TNBC subtype ($p < 0.05$), classified by ER, PR, HER2 and Ki-67 status. However, the survival curves had a similar tendency in Luminal B but not met statistic value **(Figure 2)**.

### • Elevated expression of COX5B is an independent predictive factor contributing to worse DFS

To examine whether COX5B is poor factor contributing to DFS, we analyzed the relationship between COX5B expression and DFS by univariate and multivariate methods. As shown in **Table 3**, tumor size (hazard ratio [HR]: 1.303; 95% CI: 1.067–1.590; p = 0.019), lymph node status (HR: 1.222; 95% CI: 1.034–1.446; p = 0.009) and TNM stage (HR: 1.329; 95% CI: 1.094–1.615; p = 0.004) were associated with a higher risk of recurrence and reached significance. However, when multivariate analysis was performed to evaluate whether those factors screened by univariate analysis were independent factor for worse DFS, we found that only high COX5B expression (HR: 1.999; 95% CI: 1.025–3.908; p = 0.042) and lymph node status (HR: 2.343; 95% CI: 1.074–5.110; p = 0.032) exhibited a similar trend with univariate analysis, which means that COX5B is an independent factor for poor DFS and patients with high COX5B expression were more likely to suffer tumor recurrence.

## Discussion

Mitochondria, may be the second active and intricate organelle in cell, are recognized as 'power house' where ATP or other indirect energy substance for the whole cell-life process was produced. Recently, emerging evidence shows that mitochondria dysfunctions can contribute to tumor through multifaceted interactions including defects in mitochondrial respiration chains, alterations of mitochondrial quantity or biosynthesis and mutants in genes of tricarboxylic acid cycle enzymes, etc [23,24]. However, scarce of studies focus on mitochondrial proteins encoded by nuclear genes function in breast cancer. Actually, mitochondrial proteins are encoded by two sets of genomes mitochondrial and nuclear genome, the former just encodes three proteins constructing respiratory chain complexes while proteins from the latter genomes take absolute advantage in participating mitochondria structure assembling and functions like COX5B [25].

In our previous study, we found mitochondrial protein COX5B encoded by nuclear gene, one component of COX or the complex IV, was increased in breast cancer compared with its corresponding normal tissues [26]. And the Kaplan–Meier plotter also hints that *COX5B* may act as oncogene in breast cancer since patients with higher *COX5B* expression have a worse DFS than those with lower *COX5B* expression. However, the predictive prognosis value of COX5B above is based on mRNA level [27]. Thus, IHC, the more standard and prevalent method, needs to be performed in breast cancer tissues to examine whether COX5B is associated with patients' prognosis. So, we first tested specificity of COX5B antibody in breast cancer several cell lines. The confirmed results showed that COX5B could only detect one band near 15 KD in the whole PVDF membrane without any other bands **(Figure 3)**. As far as we know, *COX5B* gene is a nuclear gene and is consisted of five exons and four introns and encodes about 15 KD mitochondrial protein [28]. Thus, the only band near 15 KD displayed in polyvinylidene fluoride (PVDF) membrane combined by COX5B antibody is exactly the COX5B protein and it can detect the correct molecular mass and sequence of COX5B antigen [26].

As expected, the results of TMA containing 244 patients' tissues of breast cancer revealed that COX5B expression is mainly focused on cytoplasm. COX5B has been proposed to be predominantly localized to the matrix face of the mitochondrial inner membrane [29,30]. So, majority of them confined to mitochondria, which are meant they localized in cytoplasm, consistent with our results detected by IHC. However, we could not confirm whether COX5B had a location in the nuclear as a few slides showed brown staining in the nuclear and the reason cannot rule out if it has a relationship with operations during the whole processes or tissue storage for more than 7 years.

In our study, stage I to III primary female breast cancer patients with IDC from 2002 to

2006 were enrolled in our center. And neoadjuvant chemotherapy (NAC) accounts for 10% with the same chemotherapy regimens, they almost equally distributed in each subtype except Luminal A. Whether the 10% NAC specimen altered the expression of COX5B, we have no evidence to verify. The most important thing is we did not collect the 10% specimen before NAC, since there is not common to do NAC during 2002–2006 in China. So, we could not measure COX5B expression before or after NAC directly. According to recent two papers on hormone receptor (ER/PR) alterations induced by NAC, PR changes were the predominant one, the discordances were reported 13, 14.5% separately, and the discordances for ER were 8.7, 12% [31,32]. COX5B positive in our study is about 40%, which is less than ER or PR positive (70%). So, we rough estimate the COX5B discordances by analogy of 10% multiplying together 14%, the 1.4% was obtained that is belong to small probability event ($p < 5\%$). In addition, we equalized NAC proportion in

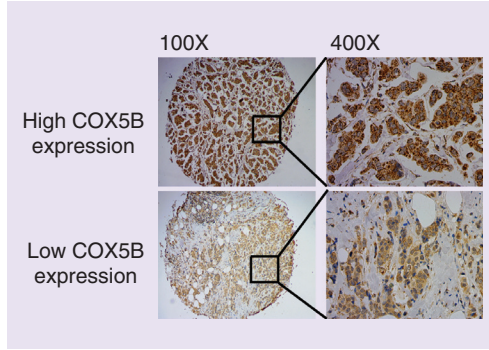


**Figure 1. Representative cytochrome c oxidase subunit VB immunohistochemical staining is presented in the large (400× magnification) and small images (100× magnification).**

**Table 2. Clinicopathological variables and the expression of cytochrome c oxidase subunit VB in this study.**

| Variables | Case (%) | COX5B expression | | p-value |
|---|---|---|---|---|
| | | Low (%) | High (%) | |
| Total | 244 | 145 (59.4) | 99 (40.6) | |
| Age: | | | | 0.974 |
| – ≤50 | 118 (48.4) | 70 (28.7) | 48 (19.7) | |
| – >50 | 126 (51.6) | 75 (30.7) | 51 (20.9) | |
| Menopausal status: | | | | 0.059 |
| – Premenopausal | 108 (44.3) | 57 (23.4) | 51 (20.9) | |
| – Postmenopausal | 136 (55.7) | 88 (36.1) | 48 (19.7) | |
| Tumor size: | | | | 0.044 |
| – ≤2 cm | 111 (45.4) | 74 (30.3) | 37 (15.1) | |
| – >2 cm | 131 (53.8) | 69 (28.2) | 62 (25.6) | |
| – Unknown | 2 (0.8) | 2 (0.8) | 0 (0) | |
| Lymph node status: | | | | 0.139 |
| – Positive | 149 (61.1) | 94 (38.5) | 55 (22.5) | |
| – Negative | 93 (38.1) | 49 (20.1) | 44 (18.0) | |
| – Unknown | 2 (0.8) | 2 (0.8) | 0 (0) | |
| ER: | | | | 0.073 |
| – Positive | 103 (42.2) | 68 (27.9) | 35 (14.3) | |
| – Negative | 141 (57.8) | 77 (31.6) | 64 (26.2) | |
| PR: | | | | 0.228 |
| – Positive | 63 (25.8) | 38 (15.6) | 25 (10.2) | |
| – Negative | 179 (73.4) | 107 (43.9) | 72 (29.5) | |
| – Unknown | 2 (0.8) | 0 (0) | 2 (0.8) | |
| HER2: | | | | 0.072 |
| – Positive | 98 (40.2) | 65 (26.6) | 33 (13.6) | |
| – Negative | 146 (59.8) | 80 (32.7) | 66 (27.1) | |

Bold was statistically significant ($p < 0.05$); Pearson's Chi-square test or *t* test, the definition of ER, PR, HER2 in this study were based on NCCN guidelines.

COX5B: Cytochrome c oxidase subunit VB; ER: Estrogen receptor; HER2: HEGF receptor 2; NCCN: National Comprehensive Cancer Network; PR: Progesterone receptor.

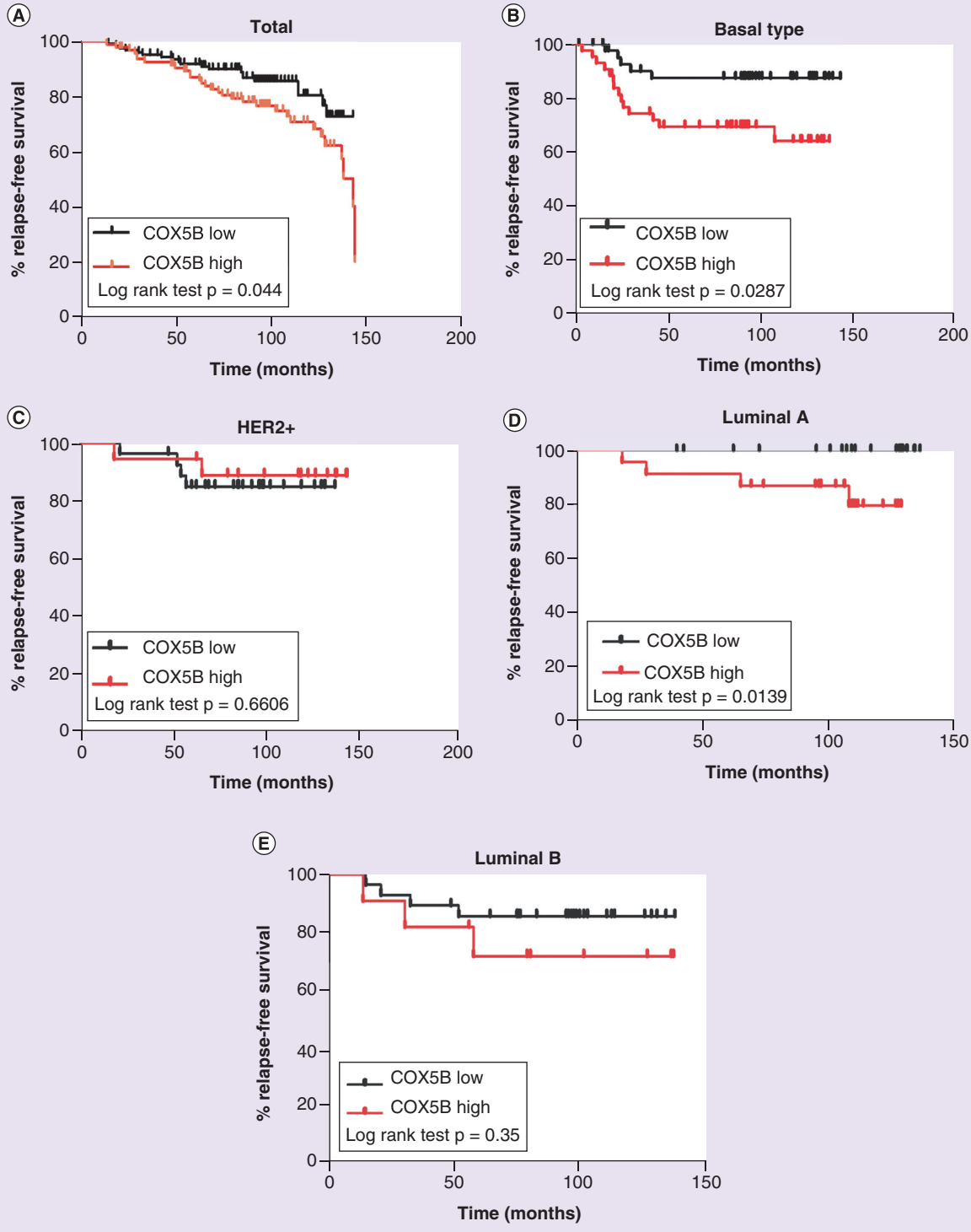


**Figure 2. Elevated expression of cytochrome c oxidase subunit VB indicates worse clinical outcome in patients with breast cancer.** **(A)** Cumulative DFS curves of breast cancer patients with low or high COX5B expression. **(B, C, D & E)** Cumulative DFS curves of breast cancer patients with low or high COX5B expression in basal type, HER2 type, Luminal A and Luminal B subgroups.
COX5B: Cytochrome c oxidase subunit VB; DFS: Disease-free survival; HER2: HEGF receptor 2.

**Table 3. Univariate and multivariate analysis for disease-free survival.**

| Variables | Univariate analysis HR (95% CI) | p-value | Multivariate analysis HR (95% CI) | p-value |
|---|---|---|---|---|
| Age | 0.827(0.477–1.434) | 0.499 | 0.426 (0.196–0.928) | |
| Menopausal status | 1.548 (0.871–2.750) | 0.136 | 2.670 (1.152–6.192) | |
| Tumor size | 1.303 (1.067–1.590) | 0.019 | 1.605 (0.904–2.850) | 0.106 |
| Lymph node status | 1.222 (1.034–1.446) | 0.009 | 2.343 (1.074–5.110) | 0.032 |
| Grade | 0.981 (0.875–1.100) | 0.745 | 0.944 (0.825–1.079) | |
| TNM stage | 1.329 (1.094–1.615) | 0.004 | 0.671 (0.354–1.270) | 0.220 |
| ER status | 0.778 (0.439–1.379) | 0.778 | 1.175 (0.500–2.759) | |
| PR status | 0.471 (0.212–1.046) | 0.064 | 0.379 (0.130–1.104) | |
| HER2 status | 0.945 (0.539–1.655) | 0.842 | 1.014 (0.513–2.005) | |
| COX5B | 1.795 (1.008–3.197) | 0.047 | 1.999 (1.025–3.908) | 0.042 |
| Radiation therapy | 0.353 (0.170–0.734) | 0.005 | 0.653 (0.257–1.657) | 0.369 |
| Chemotherapy | 1.215 (0.543–2.716) | 0.636 | 0.101 (0.451–2.690) | |

Statistically significant $p < 0.05$ both in the univariate and multivariate analyses are indicated in bold.
COX5B: Cytochrome c oxidase subunit VB; DFS: Disease-free survival; ER: Estrogen receptor; HER2: HEGF receptor 2; HR: Hazard ratio; PR: Progesterone receptor.

subgroup except Luminal A (Luminal A group is more suitable for neoadjuvant endocrine therapy) to reduce or eliminate this impact, so we think 10% NAC in our study may not change COX5B expression. And several other markers tested in this set of breast cancer already published online [33,34]. However, COX5B alterations in breast cancer specimen before or after NAC were called for further investigation.

Our results also imply that COX5B seems to be a poor factor risk for recurrence. Patients with high level of COX5B may have a worse DFS than those with low COX5B level. Furthermore, high COX5B expression is analyzed as an independent factor predictable for worse DFS by multivariate analysis, suggesting COX5B may act as an unfavorable factor in breast cancer. And when survival analyses were performed in subtype of breast cancer, we found the similar results in subtype of Luminal A and Basal like, however, there is no difference between two survival curves in HER2 and Luminal B. We could not explain why COX5B has no relationship with HER2 and Luminal B subtypes.

Besides, there have several limitations in this study, including the small dataset, long history tissue storage, the incomplete information of Ki-67 information in our cohort and the composition of the study cohort. Actually, the composition of patients in our study was not exactly representative of the composition of the breast cancer population. There is higher proportion of HER2-positive expression and lower percentage of ER- and PR-positive expression here than it did in the natural breast cancer population [35,36]. Our lad mainly focuses on metastasis of breast cancer and we put more attentions to breast cancer subtype prognosis not incidence. Fifty patients were enrolled in Luminal A, Luminal B, HER2-positive subgroup, while

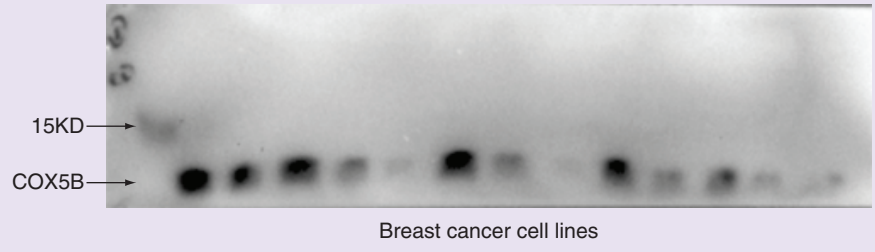


**Figure 3. Cytochrome c oxidase subunit VB expression in several breast cancer cell lines by western blot.**

100 patients were recruited in Basal type. This enrollment mode did reduce ER-, PR-positive distribution. As for HER2-positive distribution, we recruit majority of HER2-positive Luminal B rather than HER2-negative Luminal B, which resulted in almost double amount in the general population of breast cancer cases. In addition, our hospital is the top three in our country in the field of breast cancer, so the selective bias is more serious than other county hospitals. However, our study was based on the use of TMAs, which can guarantee the consistency and coherence and we put special attention on progression or metastasis leading by breast cancer, such as local or regional recurrence, so we chose DFS as our prognosis analysis system. Using this set of TMA, researcher in our lab already published several articles online [34,37]. Another larger set of TMA, with more close to general breast cancer-type distribution, are now going on its' way in our lab. We might reanalyze it in the future and we also appeal other institutes to verify COX5B functions in breast cancer or other cancers.

Collectively, our study implied that elevated COX5B expression may contribute to breast cancer. Patients with high COX5B expression might have a worse DFS than those with low expression, and additionally, high COX5B expression detected by IHC may act as an independent factor in breast cancer.

### Financial & competing interests disclosure

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*No writing assistance was utilized in the production of this manuscript.*

### Ethical conduct of research

*The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.*

## SUMMARY POINTS

- **Background**
- To evaluate cytochrome c oxidase subunit VB (COX5B) clinical implications in breast cancer based on immunohistochemistry.
- **Methods**
- Immunohistochemistry was performed on breast cancer tissue microarray.
- **Results**
- COX5B overexpression was associated with a worse disease-free survival and may act as an independent factor for worse disease-free survival in breast cancer.
- **Conclusion**
- COX5B may act as oncogene in breast cancer.

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